PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P400966 DCC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NZ 99/ 00084	14/06/1999	17/06/1998
Applicant		
NEW ZEALAND DAIRY BOARD et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NZ 99/00084

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23J3/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 06, 30 April 1998 (1998-04-30) & JP 10 033115 A (NOUCHIKUSANGIYOU SHINKO JIGYODAN;ZENKOKU NOKYO NYUGYO PLANT KYOKAI), 10 February 1998 (1998-02-10)	29
Y	abstract	1

X	PATENT ABSTRACTS OF JAPAN vol. 017, no. 086 (C-1028), 19 February 1993 (1993-02-19) & JP 04 282400 A (CALPIS FOOD IND CO LTD:THE), 7 October 1992 (1992-10-07)	29
Y	abstract	1

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1999	01/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	De Jong, E

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MULLALLY M M ET AL: "Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins." INTERNATIONAL DAIRY JOURNAL, vol. 7, no. 5, 1997, pages 299-303, XP000853725 Correspondence (Reprint) address, R. J. Fitzgerald, Dep. of Life Sci., Univ. of Limerick, Limerick, Republic of Ireland	29
Y	the whole document	1
Y	EP 0 474 506 A (MORINAGA MILK INDUSTRY CO LTD) 11 March 1992 (1992-03-11) cited in the application page 5, line 5-21; claims 1-5	1
X	ADLER-NISSEN J.: "Enzymic hydrolysis of food proteins" 1986 , ELSEVIER APPLIED SCIENCE PUBLISHERS , LONDON AND NEW YORK XP002122275	20,27,28
Y	page 59-62 page 94-96	1
A	NAKAMURA T ET AL: "ANTIGENICITY OF WHEY PROTEIN HYDROLYSATES PREPARED BY COMBINATION OF TWO PROTEASES" MILCHWISSENSCHAFT,DE,VV GMBH VOLKSWIRTSCHAFTLICHER VERLAG. MUNCHEN, vol. 48, no. 12, page 667-670 XP000432047 ISSN: 0026-3788 page 667 page 670	1-29
A	DATABASE WPI Section Ch, Week 199717 Derwent Publications Ltd., London, GB; Class D13, AN 1997-186935 XP002122276 & JP 09 047229 A (OMU NYUGYO KK), 18 February 1997 (1997-02-18) abstract	1-29
A	DATABASE WPI Section Ch, Week 199625 Derwent Publications Ltd., London, GB; Class B04, AN 1996-245843 XP002122277 & JP 08 098656 A (SNOW BRAND MILK PROD CO LTD), 16 April 1996 (1996-04-16) abstract	1-29

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NZ 99/00084

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 04593 A (TEAGASC AGRIC FOOD DEV AUTHORI) 18 March 1993 (1993-03-18) page 7, line 29 -page 8, line 35; claims 1-27	1-29
A	EP 0 065 663 A (MILES LAB) 1 December 1982 (1982-12-01) cited in the application claims 1-8	1-29

INTERNATIONAL SEARCH REPORT

[redacted]ormation on patent family members

International Application No
PCT/NZ 99/00084

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 10033115 A	10-02-1998	NONE	
JP 04282400 A	07-10-1992	NONE	
EP 0474506 A	11-03-1992	JP 2818056 B	30-10-1998
		JP 5092994 A	16-04-1993
		AU 645342 B	13-01-1994
		AU 8370491 A	12-03-1992
		CA 2050786 A,C	08-03-1992
		DE 69129395 D	18-06-1998
		DK 474506 T	02-06-1993
		NZ 239683 A	26-10-1993
		US 5304633 A	19-04-1994
JP 9047229 A	18-02-1997	NONE	
JP 8098656 A	16-04-1996	NONE	
WO 9304593 A	18-03-1993	AU 2468292 A	05-04-1993
		EP 0604467 A	06-07-1994
		IE 58923 B	01-12-1993
		NZ 244157 A	28-03-1995
EP 0065663 A	01-12-1982	JP 57194753 A	30-11-1982
		JP 58054786 B	06-12-1983

PATENT COOPERATION TR. . . .

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 February 2000 (02.02.00)	
International application No. PCT/NZ99/00084	**Applicant's or agent's file reference** P400966 DCC
International filing date (day/month/year) 14 June 1999 (14.06.99)	**Priority date** (day/month/year) 17 June 1998 (17.06.98)
Applicant SCHLOTHAUER, Ralf-Christian et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

24 December 1999 (24.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3081213

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
CALHOUN, D.
A.J. PARK & SON
Huddart Parker Building, 6th Floor,
Post Office Square, P.O. Box 949
WELLINGTON 6015
NOUVELLE-ZELANDE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	12.05.00
Applicant's or agent's file reference: P400966 DCC	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NZ99/00084	14/06/1999	17/06/1998

International Patent Classification (IPC) or both national classification and IPC
A23J3/34

Applicant
NEW ZEALAND DAIRY BOARD et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 17/10/2000.

Name and mailing address of the international preliminary examining authority:
European Patent Office - P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tx: 31 651 epo nl
Fax: +31 70 340 - 3016

Authorized officer / Examiner
De Jong, E

Formalities officer (incl. extension of time limits)
Cardenas, C
Telephone No. +31 70 340 3370

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-19 as originally filed

Claims, No.:

1-32 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 30-32,

because:

☒ the said international application, or the said claims Nos. 30-32 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	20, 27-29
Inventive step (IS)	Claims	1-29
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Ad III

1. Claims 30-32 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT, namely a method of treatment of the human body. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

Ad V and VIII

2. Reference is made to the following documents:
D1 = JP-A-10033115
D2 = JP-A-4282400
D3 = International Dairy Journal, 1997, p.299-303
D4 = EP-A-0 474 506
D5 = Enzymic hydrolysis of food proteins, 1986, p.59-62 and p.94-96
D6 = Milchwissenschaft, 1993, p.667-670

3. Claim 1 does not meet the requirements of Article 6 PCT (see also the PCT International Preliminary Examination Guidelines III 4.7) in that the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem, i.e. to produce an enzymatic whey hydrolysate containing bioactive peptides and with an acceptable flavour. See also p.2 l.36/37 and p.3 l.25-32 of the description, where it is stated that "it would be advantageous if..." and that "it is an object of the invention to go some way towards achieving these desiderata..."
The technical features necessary for achieving this result should be added.

Furthermore, because of this vague definition, the subject-matter of claim 1 is rendered obvious (Article 33(3) PCT) by teachings of the background art: as stated on p.1 l.24-30 of the present description, hydrolysis of whey proteins was known to release bioactive peptides (see D1-D4) and it was also known how DH and bitterness relate during hydrolysis (see D5).

4. The subject-matter of claims 20, 27 and 28 is anticipated (Article 33(3) PCT) by D5, see p.96.

5. Claim 29 has been drafted as another independent claim, and it is unclear if it relates effectively to the same subject-matter as the preceding claims (Rule 13 PCT). This claim therefore lacks conciseness (Article 6 PCT). It is also not clear what the difference is between the subject-matter of claims 27 and 28. Moreover, lack of clarity of the claims as a whole arises (Article 6 PCT), since it is difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.
The subject-matter of claim 29 appears to be anticipated by D1-D3.

6. The subject-matter of the dependent claims 2-19 and 21-26 is not considered to involve an inventive step. Applicant's attention is drawn to D6, wherein Neutrase is applied and to D1, where lactose is added.
If an amended independent claim, defining new and inventive subject-matter is filed, the difference of the subject-matter of the new claim vis-à-vis the state of the art and the significance thereof should be indicated. Any arguments in favour of novelty and/or inventive step should be reflected in the claim, in terms of technical features.

7. The word "about" in connection with ranges, used in claims 6, 8, 9, 13, 21 and corresponding parts of the description, is ambiguous (Article 6 PCT and see PCT International Preliminary Examination Guidelines III 4.5a).

8. The term "as herein defined" (see claim 5) leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

CALHOUN, D.
A.J. PARK & SON
Huddart Parker Building, 6th Floor,
Post Office Square, P.O. Box 949
WELLINGTON 6015
NOUVELLE-ZELANDE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	23.08.2000
Applicant's or agent's file reference P400966 DCC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NZ99/00084	14/06/1999	17/06/1998

Applicant
NEW ZEALAND DAIRY BOARD et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Cardenas, C Tel.+49 89 2399-3370

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P400966 DCC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NZ99/00084	14/06/1999	17/06/1998
International Patent Classification (IPC) or national classification and IPC A23J3/34		
Applicant NEW ZEALAND DAIRY BOARD et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 22 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
24/12/1999	23. 08. 2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer De Jong, E Telephone No. +49 89 2399 3849

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NZ99/00084

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1,2	as originally filed			
3-20	as received on	25/07/2000	with letter of	25/07/2000

Claims, No.:

1-31	as received on	25/07/2000	with letter of	25/07/2000

Drawings, sheets:

1/3-3/3	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 29-31.

because:

☒ the said international application, or the said claims Nos. 29-31 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-28
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-28
Industrial applicability (IA)	Yes:	Claims	1-28
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Ad I

1. The amendments filed with the letter dated 25.07.00 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following: claims 1-27 and especially the subject-matter of claims 1 and 5-12 (and corresponding passages in the description).

Ad III

2. Claims 29-31 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT, namely a method of treatment of the human body. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

Ad V and VIII

3. Reference is made to the following documents:
D1 = JP-A-10033115
D2 = JP-A-4282400
D3 = International Dairy Journal, 1997, p.299-303
D4 = EP-A-0 474 506
D5 = Enzymic hydrolysis of food proteins, 1986, p.59-62 and p.94-96
D6 = Milchwissenschaft, 1993, p.667-670

4. The subject-matter of the originally filed claim 1 does not meet the requirements of Article 6 PCT (see also the PCT International Preliminary Examination Guidelines III 4.7) in that the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem, i.e. to produce an enzymatic whey hydrolysate containing bioactive peptides and with an acceptable flavour. See also p.2 l.36/37 and p.3 l.25-32 of the description, where it is stated that "it would be advantageous if..." and that "it is an object of the invention to go some way towards achieving these desiderata..."
The technical features necessary for achieving this result are lacking.

Furthermore, because of this vague definition, the subject-matter of claim 1 is anticipated and rendered obvious (Articles 33(2) and 33(3) PCT) by teachings of the background art: as stated on p.1 l.24-30 of the present description, hydrolysis of whey proteins was known to release bioactive peptides (see D1-D4) and it was also known how DH and bitterness relate during hydrolysis (see D5).

5. Claim 28 has been drafted as another independent claim, and it is unclear if it relates effectively to the same subject-matter as the preceding claims (Rule 13 PCT). This claim therefore lacks conciseness (Article 6 PCT).
Moreover, lack of clarity of the claims as a whole arises (Article 6 PCT), since it is difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of the protection.
The subject-matter of claim 28 appears to be anticipated by D1-D3.

6. The subject-matter of the originally filed dependent claims 2-19 and 21-26 is not considered to involve an inventive step. Applicant's attention is drawn to D6, wherein Neutrase is applied and to D1, where lactose is added.

7. The word "about" in connection with ranges, used in claims 1, 13 and 21 and corresponding parts of the description, is ambiguous (Article 6 PCT and see PCT International Preliminary Examination Guidelines III 4.5a).

8. The term "as herein defined" (see claim 3) leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

peptides and free amino acids, thereby allowing the original bland taste of the milk proteins substrates to be retained.

Some bioactive peptides - in particular the antihypertensive peptides - are relatively stable during protein hydrolysis and are released very early during the hydrolysis of the milk protein substrate as shown in Figure 1.

The bitter flavours of milk protein hydrolysates can be improved by adding sugars or by hydrolysing natural sugars, such as lactose, already present in the milk protein substrate (Bernal and Jelen, 1989). For example sour wheys and cheese wheys are made more palatable when they have been sweetened by β-galactosidase and lactase hydrolysis of lactose (FR 2309154; US 4358464; JP 8056568).

In order to achieve a high flavour acceptability for a hydrolysed protein product which contains bioactive peptides, precise control of hydrolysis is required to prevent bitterness occurring.

A common method of termination of hydrolysis is by deactivation of the enzymes, usually by thermal deactivation at high temperatures, typically > 90-100°C for an extended period of time. However, this method cannot be used to stop the hydrolysis of whey proteins as any intact unhydrolysed whey proteins remaining in the mixture would denature and precipitate making the final product less soluble and less acceptable for the use as a food ingredient.

It would be advantageous if a process of hydrolysing whey protein could be controlled so that it directly produced a hydrolysate comprising bioactive peptides for incorporation into functional foods which did not taste bitter and where the enzyme inactivation steps did not compromise the integrity of the intact proteins in the final product.

It is an object of the invention to go some way towards achieving these desiderata or at least to offer the public a useful choice.

SUMMARY OF THE INVENTION

Accordingly the invention may be said broadly to consist in a process for preparing a whey protein hydrolysate containing bioactive peptides comprising hydrolysing a whey protein-containing substrate with one or more enzymes characterised in that:

i) the enzyme is a heat labile protease,

ii) the hydrolysis is conducted at a temperature of between about 30°C and 70°C at a pH of about 6 to about 8.5 when said enzyme is a neutral protease, and at a pH of about 3.5 to about 5.0 where said enzyme is an acid protease,

iii) the hydrolysis is terminated when a degree of hydrolysis of no greater than 15% has been reached,

iv) the hydrolysis is terminated by deactivating said one or more enzymes, and

v) the conditions for said step iv) are sufficiently mild to avoid substantial denaturation peptides or residual proteins in said hydrolysate.

Preferably said substrate is sweet whey or sweet whey protein concentrate.

Preferably the enzyme is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000.

Preferably said enzyme deactivating step iv) comprises heat deactivation.

Preferably said heat deactivation comprises heating said hydrolysate for up to ten seconds to a temperature up to 95°C.

Preferably said hydrolysis has been conducted at a temperature of below 65°C, and said heat deactivating step is conducted at 65°C to 70°C for from 10 seconds to 15 minutes.

Preferably said hydrolysis has been conducted at a temperature below 60°C, and said heat deactivating step is conducted at 60° to 65°C for from 10 seconds up to 30 minutes.

Preferably said enzyme deactivating step comprises altering the pH of said whey protein-containing substrate to a pH at which said protease is not active.

Preferably said enzyme deactivating step includes heat deactivation as defined above.

Alternatively said enzyme deactivating step iv) comprises subjecting said hydrolysate to ultrafiltration with an ultrafiltration membrane having a nominal molecular weight cutoff in the range of 10-500kDa.

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Alternatively said enzyme is immobilised on an inert support during said hydrolysis step ii).

Preferably said inert support is Roehn Eupergit, carrageenan particles, chitosan particles or any other suitable inert support material.

Preferably the degree of hydrolysis is from about 3% to about 5%.

Preferably the substrate also contains lactose, in an amount of up to 50% by weight.

Alternatively, the substrate also contains lactose in an amount of up to 30% by weight.

Preferably the substrate is also treated with lactase and/or β-galactosidase, either before or during the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

In another embodiment the invention consists in a whey protein hydrolysate containing one or more bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

The enzyme hydrolysis step may be carried out under conditions which are suitable for the particular enzyme used as would be understood by a person skilled in the art.

The whey protein substrates are hydrolysed at a concentration in the range from 5-50% solids and the enzyme or enzyme mixtures are added to give an enzyme to substrate ratio between 0.01% and 3% w/w total solids, preferably between 0.01% and 1.0% w/w total solids.

Protein substrates treated with acid proteases may be hydrolysed at pH between 2.5 and 6.0, preferably between pH 3.0 and 5.0.

Protein substrates treated with neutral proteases may be hydrolysed at pH between 3.5 and 9.0, preferably between pH 6.0 and 8.0.

Protein substrates treated with alkaline proteases may be hydrolysed at pH range between 5 and 10.0, preferably between pH 6.0 and 8.0.

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The protein hydrolysis may be carried out at a temperature range of from 30-65°C, preferably from 50-60°C.

The hydrolysis of lactose may be carried out at a prior stage to the whey protein hydrolysis, concurrently therewith or subsequently. The enzymes used for lactose hydrolysis may comprise lactase and/or β-galactosidase and may be selected from yeast or fungal sources eg *Klyvermyces lactis*, *Saccharomyces lactis*, *Saccharomyces fragillis*, eg *Aspergillus niger*, *Aspergillus oryzae* such as Maxilact (Gist Brocades) and Novolact (Novo Nordisk). The lactose hydrolysis is carried out under conditions which would be known to persons skilled in the art.

In one embodiment termination of the hydrolysis is achieved by deactivating the one or more whey protein hydrolysis enzymes (and/or the lactose hydrolysing enzymes added previously) by firstly changing the pH of the reaction mixture to a pH in which the enzyme(s) is either inactive or less active, and/or heating the reaction mixture to a comparatively mild temperature using a heat exchanger to denature the enzyme but not the intact whey proteins in the substrate. A suitable temperature range which would denature the enzymes is in the order of 55-70°C, preferable 65°C.

According to one option, depending on the enzyme(s) used, the enzyme or enzyme mixture may also be deactivated by the evaporation and drying procedures.

According to another option the enzyme or enzyme mixture may also be deactivated with or without a prior pH change.

Alternatively, the one or more enzymes used to selectively hydrolyse the whey protein may be immobilised on an inert support such as Roehm Eupergit, Carrageenan particles, chitosan particles or any other suitable material and then used in a stirred tank or fixed bed reactor or on a membrane or on a hollow fiber reactor.

Alternatively, the enzyme(s) to be used for the hydrolysis could be cross linked to suitable inert support prior to the hydrolysis reaction and subsequently separated out of the hydrolysis reaction with the use of a microfiltration membrane.

Alternatively, the enzyme can be separated away from the hydrolysis mixture with the use of an ultrafiltration membrane with a nominal molecular weight cutoff in the range 10 - 500 kDa once hydrolysis is complete.

After hydrolysis and optional deactivation or removal of enzymes, the hydrolysate may optionally be subjected to reverse osmosis under conditions whereby salt and water are removed from the hydrolysate. The purified desalted hydrolysate comprising whey proteins and bioactive peptides is then recovered. If lactose hydrolysis is also chosen then the hydrolysate will also contain glucose, galactose and/or galacto-oligosaccharides.

Optionally the hydrolysed whey proteins containing the bioactive peptide fraction can be separated with a UF membrane of 5-200 kDa cut off, preferably 10-50 kDa cut off. The bioactive peptides, other peptides and, optionally, hydrolysed lactose is recovered in the permeate.

According to another option ion exchange or hydrophobic adsorption or hydrophobic interaction chromatography or combinations of these processes may be used to recover the hydrolysed bioactive fraction from the hydrolysates in an enriched form.

In addition, lactase and β-galactosidase hydrolysis of lactose produces galacto-oligosaccharides which are known to stimulate the growth of beneficial gut flora thereby adding to the bioactive properties of the hydrolysates.

Hydrolysates which have been treated to further hydrolyse lactose are useful as food additives for consumers who are lactose intolerant.

The hydrolysed whey protein product of the invention has one or more of the following features:

- antihypertensive ACE-I activity
- bifidus growth promoting activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste
- good organoleptic properties.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described with reference to the accompanying drawings in which:

Figure 1 is a plot of bitterness and bioactivity on the ordinant against the degree of hydrolysis on the abscissa. The 'opportunity window' of obtaining a product according to the present invention containing bioactive peptides and having acceptable flavours before the hydrolysis reaction produces bitter peptides is between the lines x_1 and x_2.

Figure 2 is a plot of systolic blood pressure of four groups of hypertensive rats fed different diets over a period of eight weeks.

Figure 3 is a plot of a least squares means analysis of rats fed with a control of commercial rat chow against groups of rats fed with hydrolysate at two different concentrations per day.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention provides a process for producing a hydrolysed whey protein product containing bioactive peptides, whereby the hydrolysis is carried out under a high degree of control to prevent undesirable flavours developing during hydrolysis (eg bitter, soapy and brothy). The hydrolysis is terminated within the "opportunity window", ie before the emergence of substantial bitterness - as shown in Figure 1 - to provide hydrolysates having good organoleptic properties and maximum bioactive peptides. In Figure 1 the degree of hydrolysis is represented qualitatively on the x axis. The window of opportunity is between the points x_1 and x_2 which will vary depending on the enzyme which is used. The optimum conditions sought are a maximum bioactivity with an acceptable level of bitterness.

In particularly preferred embodiments of the process of the invention, the enzyme which hydrolyses the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined) and the hydrolysis of the whey proteins is terminated by heat treatment for a short time at a high temperature (about 85-100°C for 1-10 seconds). The applicants have surprisingly found that the above enzymes (1) are able to produce a whey protein hydrolysate containing a good level of bioactive peptides, and (2) can be inactivated by a short time, high temperature treatment which causes only partial denaturation of the whey proteins in the hydrolysate, and surprisingly improves the organoleptic properties of the whey proteins, in terms of providing a product which is creamy in texture (has a relatively small particle size) and substantially white in appearance.

The present invention is now exemplified by the following examples:

Example 1

A 10% solution of a sweet whey protein concentrate with 80% protein content (ALACEN™ 392, 2L) was hydrolysed at 50°C with the commercially available enzyme Neutrase sourced from *Bacillus subtilis* (Novo Nordisk, Denmark). A pH of 7.0 and an enzyme substrate ratio of 0.3% w/w was used for the hydrolysis. The hydrolysate was adjusted to pH 5.0 and heated at 65°C for 30min to inactivate the enzyme. The hydrolysate (DH of 4.5%) was spray dried and tested for angiotensin-converting enzyme inhibitor (ACE-I) activity and flavour. ACE-I activity in the dried product was determined using FAPGG as a substrate (Product 305-10 ex Sigma Chemical Corporation, St Louis, MO, USA) according of the method of D W Cushman & H S Cheung (1971). ACE-I activities are expressed as the amount of material (g/L) needed to reduce the activity of the ACE-I enzyme by 50%. IC_{50} ACE-I activity in the hydrolysate was 0.44g/L and flavour acceptability score, as determined by a taste panel, was very high.

Example 2

A 50% solution of ALACEN™ 421 whey protein concentrate (56% protein content, 10L) was treated with commercial lactase sourced from *Kluveromyces lactis* (Lactozyme 3000L ex Novo Nordisk) at an enzyme to substrate ratio of 0.3% at 50°C for 2 hours. The lactase treated solution was hydrolysed with Neutrase (Novo Nordisk, Denmark) for 1 hour at 50°C at an enzyme substrate ratio of 0.3%. Active enzymes were inactivated by UHT treatment (5sec at 95°C) after a five fold dilution of the mixture. The hydrolysate was spray dried. The dry powder (DH 2.8%) contained no traces of active enzyme and had an ACE-I activity of 2.18g/L. The flavour score was exceptionally high due to the introduction of a low level of sweetening into the product. ACE-I measurements and flavour acceptability scoring were determined as for Example 1.

Example 3

A 500L hydrolysate, made from ALACEN™ 392 in a similar way to that in example 1, was cooled to 10°C after enzyme inactivation. A sub-sample of the original hydrolysate was dried. The remaining hydrolysate was subjected to ultrafiltration at 10°C with a 10,000 dalton nominal molecular weight cutoff membrane (HFK 131, Koch Membrane Systems, USA). The hydrolysate (at a DH between 3.8% and 4.2%) was concentrated to

a VCF 10 and the retentate was dried directly. The permeate was concentrated by evaporation to approx 25% solids and dried. ACE-I measurement and flavour acceptability scoring were determined as for Example 1. The ACE-I activity was enriched in the permeate powder (IC_{50} of the permeate powder was 0.15g/L). ACE-I activity in the sub-sample of the dried hydrolysate before ultrafiltration was 0.43g/L. The flavour acceptability scores on the retentate powder and the spray dried powder of the feed were both high.

Example 4

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up at 15% solids to yield 150 L. The solution was treated with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) and a commercial lactase from *Klyvermyces lactis* (Lactozyme 3000L ex Novo Nordisk). The addition rate of enzyme was 0.3% w/w (on protein basis) for Neutrase and 1.2% w/w (on lactase basis) for Lactozyme. The reaction continued for 2h at 50°C at a pH of 7.0. Samples of 35L were taken every 0.5h inactivated at 88°C for 3 seconds and subsequently spray dried. The ACE-I activity as specified in example 1 yielded 0.424g/L, 0.336g/L and 0.432g/L for the three mixtures on a protein basis. The bitterness of the samples from ALACEN™ 392 was formally evaluated against two standard hydrolysates. The scores for bitterness on a scale of 1 to 10, 10 being most bitter were 1.9 for a sample after 0.5h hydrolysis, 2.3 for the 2h hydrolysis compared to 5 and 7 for the standard hydrolysis samples of greater degrees of hydrolysis.

The samples of ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose taken after 2h had a mean particle size of 3µm or 2µm respectively. The sample of ALACEN™ 392 had a mean particle size of 6µm after 2h hydrolysis and inactivation as specified. Less grittiness and chalkiness was attributed to the smaller particle size samples.

The solubility of the hydrolysed ALACEN™ 392/lactose mixture was the highest with approximately 85% across the pH range. The ALACEN™ 392, ALACEN™ 421 samples are soluble to about 70% with a slight drop in solubility to 65% at pH 3.5.

Example 5

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up of 30% solids to yield 75L. The enzyme

treatment was done using the same conditions as example 4. The samples taken at half hourly intervals were diluted to 15% solids. Otherwise the heat treatment was done as in example 4. The ACE-I activity measured as specified in example 1 was 0.560g/L, 0.440g/L and 0.728g/L.

Samples from example 4 and 5 were added in a concentration of 0.1% to the standard growth media of *Bifidobacterium lactis* and resulted in a faster cell growth and higher final cell density of the strain than the control without any supplement.

The oligosaccharide level (trisaccharides and higher) of those three hydrolysed samples was 0.2%, 2.1% and 7.0% in ALACEN™ 392, ALACEN™ 421 and the mixture of ALACEN™ 392 and lactose, respectively.

Example 6

Hydrolysate powders prepared in example 5 were used as a supplement for yoghurts in addition rates from 2.5% and 5% of the final yoghurt and resulted in an increased creaminess and improved the texture compared to the control.

Example 7

The hydrolysate powders prepared in example 5 were used as the protein source in a muesli bar recipe on a 6% and 12% w/w addition rate. All tasters preferred the hydrolysate bars over the unhydrolysed WPC control. The best results were achieved with hydrolysed ALACEN™ 421 and a mixture of ALACEN ™392 and lactose prepared in example 5.

Example 8

The hydrolysate powder prepared in example 5 was used as an ingredient in a meal replacer concept sample. ALACEN™ 421 hydrolysed in lactose and protein was added at a rate of 45% w/w to whole milk powder, malto dextrin, sucrose and milk calcium (ALAMIN™) to result in a powder meal replacer prototype. In comparison with a control sample without hydrolysed whey protein, hydrolysed whey protein prepared in example 5 was found to be more acceptable.

Example 9

A nutritional whey protein drink was formulated containing 8% w/w of ALACEN™ 392 or ALACEN™ 421 or a mixture of ALACEN™ 392 and lactose hydrolysed as specified in example 5. The drink also contained sucrose, citric acid, flavouring and colouring agents. The pH of the drink was adjusted to 4.3. The drink combined the nutritional and health advantages of whey protein with the refreshing taste of a soft drink. Compared to a drink containing untreated whey protein control the pH stability was improved and the drink had a more milk like appearance than the control.

Example 10

A further nutritional protein drink was formulated containing 12.5% w/w of ALACEN™ 421 hydrolysed as in example 5 in water mixed with pasteurised whole milk. Sucrose was added to yield 6% of the final formulation as well as stabiliser. The drink was flavoured when desired with banana, vanilla or similar flavours. To achieve an extended shelf life the drink was ultra high heated to 140°C for 3 seconds. The mean particle size remains at 3 microns after the additional UHT heat treatment.

Example 11

The hydrolysis was carried out as specified in example 5 but instead of reconstituting ALACEN™ 421 powder a fresh retentate of ALACEN™ was concentrated to 30% solids in the solution. The neutrase addition rate was varied to 0.9% w/w (on a protein base), the lactase level as specified. The reaction mixture was inactivated at 15% solids after 2h. The ACE-I activity yielded 0.480g/L. The organoleptic properties, particle size and food application were very similar to example 4 and 5.

Example 12

The hydrolysis was carried out as specified in example 4 with ALACEN™ 421 powder. The Neutrase addition rate was varied to 0.9% w/w (on a protein basis). The lactose was converted with a lactase from *Aspergillus oryzae* (Fungal lactase 30,000, Kyowa Enzymes Co. Ltd. Japan) on an addition rate of 0.4% w/w (on lactose base). The reaction mixture was inactivated after 1.5h with direct steam injection to achieve a temperature of 88°C for either 1.5 seconds or 3 seconds.

The particle size was 2.3 microns. Organoleptic properties and food application were very similar to the product of example 4.

Example 13

A 10% w/w solution of ALACEN™ 392 was hydrolysed with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) at an enzyme concentration of 0.9% w/w. The reaction continued for 6h at 50°C. Samples of 200ml were taken every 1h, inactivated at 88°C for 8 seconds and subsequently freeze dried.

ACE-I activity, degree of hydrolysis, pH of solution and bitterness developed over time as follows. The higher the bitterness score the more bitter is the taste. The smaller the level measured, the higher is the ACE-I activity.

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TABLE 1: Hydrolysis of ALACEN™ 392 WPC

Hydrolysis time [h]	ACE-I activity [g/L] (IC_{50})	Degree of hydrolysis [%]	pH of solution	Bitterness score [informal, 0-10]
1	0.420	3.86	7.01	0
2	0.280	3.78	6.96	1
3	0.230	4.53	6.92	1
4	0.220	4.89	6.89	3.5
5	0.210	5.20	6.87	2
6	0.190	5.37	6.87	4.5

Example 14

A 10% w/w solution of ALACEN™ 392 was hydrolysed with the following commercial proteases at 1% w/w, 50°C for 1h. The reaction was inactivated at 88°C for 8 seconds and subsequently the hydrolysate was freeze dried.

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TABLE 2: Hydrolysis with Different Enzymes

Enzyme	ACE-I activity [g/L](IC_{50})	pH	Degree of hydrolysis [%]
Protease P6, neutral protease, *Aspergillus* strains, Amano Enzymes	0.274	7.0	8.9
Protease A, neutral protease, *Aspergillus oryzae*, Amano Enzymes	0.443	7.0	9.2
Protease M, acid protease, *Aspergillus oryzae*, Amano Enzymes	0.450	4.0	7.4
Peptidase, neutral peptidase, *Aspergillus oryzae*, Amano Enzymes	0.540	7.0	6.9
Neutrase, neutral bacterial protease, *Bacillus subtillis*, Novo Nordisk DK	0.510	7.0	4.3
Validase (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.510	4.0	5.6
AFP 2000 (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.550	4.0	3.9

Example 15

Identification of ACEI-Peptides and Measuring their Activities

200 mg of permeate from example 3 was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a Jupiter preparative reverse-phase HPLC column (10 micron, C18, 22 x 250 mm [Phenomenex NZ]) equilibrated with solvent A (0.1%TFA) and connected to an FPLC system (Pharmacia). Peptides were sequentially eluted from the column with a gradient of 0 to 43% solvent B (0.08% TFA in acetonitrile) in 245 min at a flow rate of 10mL/min. Peptides eluting from the column were detected by monitoring the absorbance of the eluate at 214nm. The eluate was collected by an automatic fraction collector set to collect 3 min fractions.

Each fraction was lyophilised and the amount of peptide material present was measured gravimetrically. Fractions were assayed for ACE-I activity using an *in vitro* assay system (reagents from Sigma product 305-10) consisting of rabbit lung ACE and the

colorimetric ACE substrate furylacryloylphenylalanylglycylglycine (FAPGG); ACE hydrolyses FAPGG to give the products FAP and GG which results in a decrease in absorbance at 340mm. If a peptide inhibits ACE, the change in absorbance at 340nm is reduced. Fractions containing the highest ACE inhibitory activity per mg peptide material were re-applied to the preparative reverse-phase HPLC column and eluted using a shallow gradient of solvent B *i.e.* 0.09% increase in solvent B concentration/min. The eluate was collected using the fraction collector set to collect 0.5 min fractions.

Samples from each fraction were analysed using an analytical reverse-phase HPLC column, and those fractions containing a single, identical peptide were pooled. Each pooled fraction was lyophilised and the weight of the peptide present was determined gravimetrically. The purified peptides were assayed for ACE-I activity as before and the IC_{50} was calculated for each individual peptide.

The molecular weight of each peptide was determined by Electrospray Ionisation Mass Spectrometry (Sciex API 300 triple quadrupole mass spectrometer). Tandem mass spectrometry was also done for each peptide to generate CAD spectra using MSMS experiment scans. Each peptide was also analysed by an automated N-terminal sequencer (ABI model 476A protein sequencer). Data collected from all three techniques was used to deduce the sequence of all of the peptides possessing ACE-I activity. The origin of each of the active peptides was determined by searching a database containing the known sequences of all bovine milk proteins.

The peptides, their origins, activities and known similarities are set out in table 3. Although the last three peptides are of a casein origin they were from a whey protein hydrolysate. The rennet used to precipitate casein did not precipitate these casein fractions and they remained with the whey proteins.

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TABLE 3: ACE-I Peptides and their Activities

Peptide Sequence[a]	Origin	Activity[b] (IC_{50} in mg L^{-1})	Similarity to known ACE-I Peptides
AFE (Ala-Phe-Glu)	PP[d]3(129-131)	20	
LFSH (Leu-Phe-Ser-His)	PP3(125-128)	30	
ILKEKH (Ile-Leu-Lys-Glu-Lys-His)	PP3(71-76)	20	
LIVTQ (Leu-Ile-Val-Thr-Gln)	β-LG[e](1-5)	17	
MKG (Met-Lys-Gly)	β-LG(7-9)	24	
LDIQK[c] (Leu-Asp-Ile-Gln-Lys)	β-LG(10-14)	17	β-LG(9-14)
VF (Val-Phe)	β-LG(81-82)	19	
ALPMH (Ala-Leu-Pro-Met-His)	β-LG(142-146)	12	β-LG(142-148)
VTSTAV (Val-Thr-Ser-Thr-Ala-Val)	GMP[f](59-64)	30	
LHLPLP (Leu-His-Leu-Pro-Leu-Pro)	β-CN[g](133-138)	7	
LVYPFPGPIPNSLPQNIPP (Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro)	β-CN(58-76)	19	β-CN(74-76)
LFRQ (Leu-Phe-Arg-Glu)	α_{s1}-CN(136-139)	17[h]	

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[a] sequence given using the single-letter amino acid code with the corresponding three-letter code in brackets

[b] using the colorimetric substrate FAPGG

[c] most abundant ACE-I in hydrolysate

[d] protease peptone

[e] β-lactoglobulin

[f] glycomacropeptide

[g] β-casein

[h] activity measured with that of another peptide of unknown origin

Example 16

The effect of the hydrolysate powder prepared in example 3 (without ultrafiltration) on *in vivo* blood pressure was tested using spontaneously hypertensive rats (SHR/N). The rat strain has been specifically selected for their development of high blood pressure on maturing, and is used extensively to monitor the effect of blood pressure lowering agents. They were purchased from Animal Resources Centre, P O Box 1180 Canning Vale, Western Australia 6155.

Eight week old rats were individually housed in plastic rat cages and kept in temperature controlled facilities throughout the trial. They had unlimited access to water and were fed commercial rat chow *ad libitum*. The test products were given orally as a single daily dose for 8 weeks during which time changes in blood pressure were monitored. Their blood pressure was measured using a specially designed tail cuff and blood pressure monitoring apparatus (IITC Inc., Life Science Instruments, 23924 Victory Blvd, Woodland Hilld, CA 91367). The experimental design was approved by the Massey University Animal Ethics Committee, protocol number 98/141.

The changes in the systolic blood pressures of each group of animals over the eight weeks are plotted in Figure 2 (as least squares means). The hydrolysate at both 2g/Kg bodyweight/day and 4g/Kg bodyweight/day significantly lowered the systolic blood pressure of SHRs compared to animals fed commercial rat chow only ($p<0.004$ by least-squares means analysis, see Figure 3). The effect of the hydrolysate was not as great as that of captopril, a known ACE-I inhibitory drug administered at 30mg/Kg bodyweight/day, but was a significant improvement for animals fed commercial rat chow only.

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CLAIMS

1. A process for preparing a soluble whey protein hydrolysate containing bioactive peptides comprising hydrolysing a whey protein-containing substrate with one or more enzymes characterised in that
   i) the enzyme is a heat labile protease,
   ii) the hydrolysis is conducted at a temperature of between about 30°C and 70°C at a pH of about 6 to about 8.5 when said enzymes is a neutral proteases, and at a pH of about 3.5 to about 5 when said enzyme is an acid protease and 3.5 to 5.0 where said enzyme is an acid protease,
   iii) the hydrolysis is terminated when a degree of hydrolysis of no greater than 15% has been reached,
   iv) the hydrolysis is terminated by deactivating said one or more enzymes, and
   v) the conditions for said step iv) are sufficiently mild to avoid substantial denaturation peptides in said hydrolysate.

2. A process as claimed in claim 1 wherein said substrate is sweet whey or sweet whey protein concentrate.

3. A process as claimed in claim 1 or 2 wherein said enzyme is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined).

4. A process as claimed in claim 1, 2 or 3 wherein said enzyme deactivating step iv) comprises heat deactivation.

5. A process according to claim 4 wherein said heat deactivation comprises heating and hydrolysate for up to ten seconds to a temperature up to 95°C.

6. A process according to claim 4 wherein said hydrolysis has been conducted at a temperature of below 65°C, wherein said heat deactivating step is conducted at 65°C to 70°C for from 10 seconds to 15 minutes.

7. A process according to claim 4 wherein said hydrolysis has been conducted at a temperature below 60°C, wherein said heat deactivating step is conducted at 60° to 65°C for from 10 seconds up to 30 minutes.

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8. A process as claimed in claim 1, 2 or 3 wherein said enzyme deactivating step comprises altering the pH of said whey protein-containing substrate to a pH at which said protease is not active.

9. A process as claimed in claim 8 wherein said enzyme deactivating step includes heat deactivation as claimed in any one of claims 4 to 7.

10. A process as claimed in claim 1, 2 or 3 wherein said enzyme deactivating step iv) comprises subjecting said hydrolysate to ultrafiltration with an ultrafiltration membrane having a nominal molecular weight cutoff in the range of 10-500kDa.

11. A process as claimed in any one of the preceding claims wherein said enzyme is immobilised on an inert support during said hydrolysis step ii).

12. A process as claimed in claim 11 wherein said inert support is Roehn Eupergit, carrageenan particles, chitosan particles or any other suitable inert support material.

13. A process as claimed in any one of the preceding claims wherein the degree of hydrolysis is about 3-5%.

14. A process as claimed in any one of the preceding claims wherein the substrate also contains lactose, in an amount of about 5% by weight or higher.

15. A process as claimed in any one of the preceding claims wherein said lactose content is about 10% by weight or higher.

16. A process as claimed in claim 14 or 15 wherein the amount of lactose present in the substrate is up to about 30% by weight.

17. A process as claimed in claim 14 or 15 wherein the amount of lactose present in the substrate is up to about 50% by weight.

18. A process as claimed in any one of claims 14 to 17, wherein the substrate is also treated with lactase and/or β-galactosidase, either before, during or after the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

AMENDED SHEET

19. A process as claimed in any one of the preceding claims wherein the hydrolysate so prepared contains one or more of the bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

20. A non-bitter soluble whey protein hydrolysate produced by a process according to any one of claims 1 to 19.

21. A product as claimed in claim 20 wherein the degree of hydrolysis of the whey proteins is about 3 to 5%.

22. A product as claimed in either of claims 20 or 21 wherein the mean particle size of the whey proteins in the product is less than about 30 microns.

23. A product as claimed in claim 22 wherein said mean particle size is less than about 3 microns.

24. A product as claimed in any one of claims 20 to 23 which is substantially white in appearance.

25. A product as claimed in any one of claims 20 to 24 which also contains galacto-oligosaccharides.

26. A product as claimed in any one of claims 20 to 25 wherein one or more of said bioactive peptides is selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

27. A food product containing a whey protein hydrolysate as claimed in any one of claims 20 to 26.

28. Any one or any combination of two or more of the bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

29. A method of reducing systolic blood pressure in a subject which comprises administering to that subject an effective amount of a product as claimed in any one of claims 20 to 28.

30. A method as claimed in claim 29 which comprises administering an effective amount of a product according to claim 26.

31. A method as claimed in claim 29 which comprises administering an effective amount of a peptide or a combination of peptides as claimed in claim 28.

peptides and free amino acids, thereby allowing the original bland taste of the milk proteins substrates to be retained.

Some bioactive peptides - in particular the antihypertensive peptides - are relatively stable during protein hydrolysis and are released very early during the hydrolysis of the milk protein substrate as shown in Figure 1.

The bitter flavours of milk protein hydrolysates can be improved by adding sugars or by hydrolysing natural sugars, such as lactose, already present in the milk protein substrate (Bernal and Jelen, 1989). For example sour wheys and cheese wheys are made more palatable when they have been sweetened by β-galactosidase and lactase hydrolysis of lactose (FR 2309154; US 4358464; JP 8056568).

In order to achieve a high flavour acceptability for a hydrolysed protein product which contains bioactive peptides, precise control of hydrolysis is required to prevent bitterness occurring.

A common method of termination of hydrolysis is by deactivation of the enzymes, usually by thermal deactivation at high temperatures, typically > 90-100°C for an extended period of time. However, this method cannot be used to stop the hydrolysis of whey proteins as any intact unhydrolysed whey proteins remaining in the mixture would denature and precipitate making the final product less soluble and less acceptable for the use as a food ingredient.

It would be advantageous if a process of hydrolysing whey protein could be controlled so that it directly produced a hydrolysate comprising bioactive peptides for incorporation into functional foods which did not taste bitter and where the enzyme inactivation steps did not compromise the integrity of the intact proteins in the final product.

It is an object of the invention to go some way towards achieving these desiderata or at least to offer the public a useful choice.

SUMMARY OF THE INVENTION

Accordingly the invention may be said broadly to consist in a process for preparing a whey protein hydrolysate containing bioactive peptides which comprises the following steps:

(i) treating a whey protein containing substrate with one or more enzymes capable of hydrolysing whey proteins, to produce a whey protein hydrolysate containing bioactive peptides; and

(ii) terminating the hydrolysis before substantial production of bitter flavours.

Preferably the hydrolysis is terminated under conditions which cause no more than partial denaturation of the whey proteins and which maintain or improve their organoleptic properties.

More preferably the hydrolysis is terminated under conditions which partially denature the whey proteins and thereby improve their organoleptic properties.

Preferably the enzyme capable of hydrolysing the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined).

Preferably the hydrolysis is terminated by heat treatment, preferably for a period of about 1 to 10 seconds at a temperature of about 85°C to 100°C.

Preferably the degree of hydrolysis of the substrate before termination of hydrolysis is up to 10%.

More preferably the degree of hydrolysis is from about 3% to about 5%.

Preferably the substrate also contains lactose, in an amount of up to 50% by weight.

Alternatively, the substrate also contains lactose in an amount of up to 30% by weight.

Preferably the substrate is also treated with lactase and/or β-galactosidase, either before or during the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

In another embodiment the invention consists in a whey protein hydrolysate containing one or more bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

The enzyme hydrolysis step may be carried out under conditions which are suitable for the particular enzyme used as would be understood by a person skilled in the art.

The whey protein substrates are hydrolysed at a concentration in the range from 5-50% solids and the enzyme or enzyme mixtures are added to give an enzyme to substrate ratio between 0.01% and 3% w/w total solids, preferably between 0.01% and 1.0% w/w total solids.

Protein substrates treated with acid proteases may be hydrolysed at pH between 2.5 and 6.0, preferably between pH 3.0 and 5.0.

Protein substrates treated with neutral proteases may be hydrolysed at pH between 3.5 and 9.0, preferably between pH 6.0 and 8.0.

Protein substrates treated with alkaline proteases may be hydrolysed at pH range between 5 and 10.0, preferably between pH 6.0 and 8.0.

The protein hydrolysis may be carried out at a temperature range of from 20-65°C, preferably from 50-60°C.

The hydrolysis of lactose may be carried out at a prior stage to the whey protein hydrolysis, concurrently therewith or subsequently. The enzymes used for lactose hydrolysis may comprise lactase and/or β-galactosidase and may be selected from yeast or fungal sources eg *Klyvermyces lactis*, *Saccharomyces lactis*, *Saccharomyces fragillis*, eg *Aspergillus niger*, *Aspergillus oryzae* such as Maxilact (Gist Brocades) and Novolact (Novo Nordisk). The lactose hydrolysis is carried out under conditions which would be known to persons skilled in the art.

In one embodiment termination of the hydrolysis is achieved by deactivating the one or more whey protein hydrolysis enzymes (and/or the lactose hydrolysing enzymes added previously) by firstly changing the pH of the reaction mixture to a pH in which the enzyme(s) is either inactive or less active, and/or heating the reaction mixture to a comparatively mild temperature using a heat exchanger to denature the enzyme but not the intact whey proteins in the substrate. A suitable temperature range which would denature the enzymes is in the order of 55-70°C, preferable 65°C.

According to one option, depending on the enzyme(s) used, the enzyme or enzyme mixture may also be deactivated by the evaporation and drying procedures.

According to another option the enzyme or enzyme mixture may also be deactivated with or without a prior pH change.

Alternatively, the one or more enzymes used to selectively hydrolyse the whey protein may be immobilised on an inert support such as Roehm Eupergit, Carrageenan particles, chitosan particles or any other suitable material and then used in a stirred tank or fixed bed reactor or on a membrane or on a hollow fiber reactor.

Alternatively, the enzyme(s) to be used for the hydrolysis could be cross linked to suitable inert support prior to the hydrolysis reaction and subsequently separated out of the hydrolysis reaction with the use of a microfiltration membrane.

Alternatively, the enzyme can be separated away from the hydrolysis mixture with the use of an ultrafiltration membrane with a nominal molecular weight cutoff in the range 10 - 500 kDa once hydrolysis is complete.

After hydrolysis and optional deactivation or removal of enzymes, the hydrolysate may optionally be subjected to reverse osmosis under conditions whereby salt and water are removed from the hydrolysate. The purified desalted hydrolysate comprising whey proteins and bioactive peptides is then recovered. If lactose hydrolysis is also chosen then the hydrolysate will also contain glucose, galactose and/or galacto-oligosaccharides.

Optionally the hydrolysed whey proteins containing the bioactive peptide fraction can be separated with a UF membrane of 5-200 kDa cut off, preferably 10-50 kDa cut off. The bioactive peptides, other peptides and, optionally, hydrolysed lactose is recovered in the permeate.

According to another option ion exchange or hydrophobic adsorption or hydrophobic interaction chromatography or combinations of these processes may be used to recover the hydrolysed bioactive fraction from the hydrolysates in an enriched form.

In addition, lactase and β-galactosidase hydrolysis of lactose produces galacto-oligosaccharides which are known to stimulate the growth of beneficial gut flora thereby adding to the bioactive properties of the hydrolysates.

Hydrolysates which have been treated to further hydrolyse lactose are useful as food additives for consumers who are lactose intolerant.

The hydrolysed whey protein product of the invention has one or more of the following features:

- antihypertensive ACE-I activity
- bifidus growth promoting activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste
- good organoleptic properties.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described with reference to the accompanying drawings in which:

Figure 1 is a plot of bitterness and bioactivity on the ordinant against the degree of hydrolysis on the abscissa. The 'opportunity window' of obtaining a product according to the present invention containing bioactive peptides and having acceptable flavours before the hydrolysis reaction produces bitter peptides is between the lines x_1 and x_2.

Figure 2 is a plot of systolic blood pressure of four groups of hypertensive rats fed different diets over a period of eight weeks.

Figure 3 is a plot of a least squares means analysis of rats fed with a control of commercial rat chow against groups of rats fed with hydrolysate at two different concentrations per day.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention provides a process for producing a hydrolysed whey protein product containing bioactive peptides, whereby the hydrolysis is carried out under a high degree of control to prevent undesirable flavours developing during hydrolysis (eg bitter, soapy and brothy). The hydrolysis is terminated within the "opportunity window", ie before the emergence of substantial bitterness - as shown in Figure 1 - to provide hydrolysates having good organoleptic properties and maximum bioactive peptides. In Figure 1 the degree of hydrolysis is represented qualitatively on

the x axis. The window of opportunity is between the points x_1 and x_2 which will vary depending on the enzyme which is used. The optimum conditions sought are a maximum bioactivity with an acceptable level of bitterness.

In particularly preferred embodiments of the process of the invention, the enzyme which hydrolyses the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined) and the hydrolysis of the whey proteins is terminated by heat treatment for a short time at a high temperature (about 85-100°C for 1-10 seconds). The applicants have surprisingly found that the above enzymes (1) are able to produce a whey protein hydrolysate containing a good level of bioactive peptides, and (2) can be inactivated by a short time, high temperature treatment which causes only partial denaturation of the whey proteins in the hydrolysate, and surprisingly improves the organoleptic properties of the whey proteins, in terms of providing a product which is creamy in texture (has a relatively small particle size) and substantially white in appearance.

The present invention is now exemplified by the following examples:

Example 1

A 10% solution of a sweet whey protein concentrate with 80% protein content (ALACEN™ 392, 2L) was hydrolysed at 50°C with the commercially available enzyme Neutrase sourced from *Bacillus subtilis* (Novo Nordisk, Denmark). A pH of 7.0 and an enzyme substrate ratio of 0.3% w/w was used for the hydrolysis. The hydrolysate was adjusted to pH 5.0 and heated at 65°C for 30min to inactivate the enzyme. The hydrolysate (DH of 4.5%) was spray dried and tested for angiotensin-converting enzyme inhibitor (ACE-I) activity and flavour. ACE-I activity in the dried product was determined using FAPGG as a substrate (Product 305-10 ex Sigma Chemical Corporation, St Louis, MO, USA) according of the method of D W Cushman & H S Cheung (1971). ACE-I activities are expressed as the amount of material (g/L) needed to reduce the activity of the ACE-I enzyme by 50%. IC_{50} ACE-I activity in the hydrolysate was 0.44g/L and flavour acceptability score, as determined by a taste panel, was very high.

Example 2

A 50% solution of ALACEN™ 421 whey protein concentrate (56% protein content, 10L) was treated with commercial lactase sourced from *Kluveromyces lactis* (Lactozyme

3000L ex Novo Nordisk) at an enzyme to substrate ratio of 0.3% at 50°C for 2 hours. The lactase treated solution was hydrolysed with Neutrase (Novo Nordisk, Denmark) for 1 hour at 50°C at an enzyme substrate ratio of 0.3%. Active enzymes were inactivated by UHT treatment (5sec at 95°C) after a five fold dilution of the mixture. The hydrolysate was spray dried. The dry powder (DH 2.8%) contained no traces of active enzyme and had an ACE-I activity of 2.18g/L. The flavour score was exceptionally high due to the introduction of a low level of sweetening into the product. ACE-I measurements and flavour acceptability scoring were determined as for Example 1.

Example 3

A 500L hydrolysate, made from ALACEN™ 392 in a similar way to that in example 1, was cooled to 10°C after enzyme inactivation. A sub-sample of the original hydrolysate was dried. The remaining hydrolysate was subjected to ultrafiltration at 10°C with a 10,000 dalton nominal molecular weight cutoff membrane (HFK 131, Koch Membrane Systems, USA). The hydrolysate (at a DH between 3.8% and 4.2%) was concentrated to a VCF 10 and the retentate was dried directly. The permeate was concentrated by evaporation to approx 25% solids and dried. ACE-I measurement and flavour acceptability scoring were determined as for Example 1. The ACE-I activity was enriched in the permeate powder (IC_{50} of the permeate powder was 0.15g/L). ACE-I activity in the sub-sample of the dried hydrolysate before ultrafiltration was 0.43g/L. The flavour acceptability scores on the retentate powder and the spray dried powder of the feed were both high.

Example 4

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up at 15% solids to yield 150 L. The solution was treated with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) and a commercial lactase from *Klyvermyces lactis* (Lactozyme 3000L ex Novo Nordisk). The addition rate of enzyme was 0.3% w/w (on protein basis) for Neutrase and 1.2% w/w (on lactase basis) for Lactozyme. The reaction continued for 2h at 50°C at a pH of 7.0. Samples of 35L were taken every 0.5h inactivated at 88°C for 3 seconds and subsequently spray dried. The ACE-I activity as specified in example 1 yielded 0.424g/L, 0.336g/L and 0.432g/L for the three mixtures on a protein basis. The bitterness of the samples from ALACEN™ 392 was formally evaluated against two standard hydrolysates. The scores for bitterness on a scale of 1 to 10, 10 being most

bitter were 1.9 for a sample after 0.5h hydrolysis, 2.3 for the 2h hydrolysis compared to 5 and 7 for the standard hydrolysis samples of greater degrees of hydrolysis.

The samples of ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose taken after 2h had a mean particle size of 3µm or 2µm respectively. The sample of ALACEN™ 392 had a mean particle size of 6µm after 2h hydrolysis and inactivation as specified. Less grittiness and chalkiness was attributed to the smaller particle size samples.

The solubility of the hydrolysed ALACEN™ 392/lactose mixture was the highest with approximately 85% across the pH range. The ALACEN™ 392, ALACEN™ 421 samples are soluble to about 70% with a slight drop in solubility to 65% at pH 3.5.

Example 5

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up of 30% solids to yield 75L. The enzyme treatment was done using the same conditions as example 4. The samples taken at half hourly intervals were diluted to 15% solids. Otherwise the heat treatment was done as in example 4. The ACE-I activity measured as specified in example 1 was 0.560g/L, 0.440g/L and 0.728g/L.

Samples from example 4 and 5 were added in a concentration of 0.1% to the standard growth media of *Bifidobacterium lactis* and resulted in a faster cell growth and higher final cell density of the strain than the control without any supplement.

The oligosaccharide level (trisaccharides and higher) of those three hydrolysed samples was 0.2%, 2.1% and 7.0% in ALACEN™ 392, ALACEN™ 421 and the mixture of ALACEN™ 392 and lactose, respectively.

Example 6

Hydrolysate powders prepared in example 5 were used as a supplement for yoghurts in addition rates from 2.5% and 5% of the final yoghurt and resulted in an increased creaminess and improved the texture compared to the control.

Example 7

The hydrolysate powders prepared in example 5 were used as the protein source in a muesli bar recipe on a 6% and 12% w/w addition rate. All tasters preferred the hydrolysate bars over the unhydrolysed WPC control. The best results were achieved with hydrolysed ALACEN™ 421 and a mixture of ALACEN ™392 and lactose prepared in example 5.

Example 8

The hydrolysate powder prepared in example 5 was used as an ingredient in a meal replacer concept sample. ALACEN™ 421 hydrolysed in lactose and protein was added at a rate of 45% w/w to whole milk powder, malto dextrin, sucrose and milk calcium (ALAMIN™) to result in a powder meal replacer prototype. In comparison with a control sample without hydrolysed whey protein, hydrolysed whey protein prepared in example 5 was found to be more acceptable.

Example 9

A nutritional whey protein drink was formulated containing 8% w/w of ALACEN™ 392 or ALACEN™ 421 or a mixture of ALACEN™ 392 and lactose hydrolysed as specified in example 5. The drink also contained sucrose, citric acid, flavouring and colouring agents. The pH of the drink was adjusted to 4.3. The drink combined the nutritional and health advantages of whey protein with the refreshing taste of a soft drink. Compared to a drink containing untreated whey protein control the pH stability was improved and the drink had a more milk like appearance than the control.

Example 10

A further nutritional protein drink was formulated containing 12.5% w/w of ALACEN™ 421 hydrolysed as in example 5 in water mixed with pasteurised whole milk. Sucrose was added to yield 6% of the final formulation as well as stabiliser. The drink was flavoured when desired with banana, vanilla or similar flavours. To achieve an extended shelf life the drink was ultra high heated to 140°C for 3 seconds. The mean particle size remains at 3 microns after the additional UHT heat treatment.

Example 11

The hydrolysis was carried out as specified in example 5 but instead of reconstituting ALACEN™ 421 powder a fresh retentate of ALACEN™ was concentrated to 30% solids in the solution. The neutrase addition rate was varied to 0.9% w/w (on a protein base), the lactase level as specified. The reaction mixture was inactivated at 15% solids after 2h. The ACE-I activity yielded 0.480g/L. The organoleptic properties, particle size and food application were very similar to example 4 and 5.

Example 12

The hydrolysis was carried out as specified in example 4 with ALACEN™ 421 powder. The Neutrase addition rate was varied to 0.9% w/w (on a protein basis). The lactose was converted with a lactase from *Aspergillus oryzae* (Fungal lactase 30,000, Kyowa Enzymes Co. Ltd. Japan) on an addition rate of 0.4% w/w (on lactose base). The reaction mixture was inactivated after 1.5h with direct steam injection to achieve a temperature of 88°C for either 1.5 seconds or 3 seconds.

The particle size was 2.3 microns. Organoleptic properties and food application were very similar to the product of example 4.

Example 13

A 10% w/w solution of ALACEN™ 392 was hydrolysed with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) at an enzyme concentration of 0.9% w/w. The reaction continued for 6h at 50°C. Samples of 200ml were taken every 1h, inactivated at 88°C for 8 seconds and subsequently freeze dried.

ACE-I activity, degree of hydrolysis, pH of solution and bitterness developed over time as follows. The higher the bitterness score the more bitter is the taste. The smaller the level measured, the higher is the ACE-I activity.

TABLE 1: Hydrolysis of ALACEN™ 392 WPC

Hydrolysis time [h]	ACE-I activity [g/L] (IC_{50})	Degree of hydrolysis [%]	pH of solution	Bitterness score [informal, 0-10]
1	0.420	3.86	7.01	0
2	0.280	3.78	6.96	1
3	0.230	4.53	6.92	1
4	0.220	4.89	6.89	3.5
5	0.210	5.20	6.87	2
6	0.190	5.37	6.87	4.5

Example 14

A 10% w/w solution of ALACEN™ 392 was hydrolysed with the following commercial proteases at 1% w/w, 50°C for 1h. The reaction was inactivated at 88°C for 8 seconds and subsequently the hydrolysate was freeze dried.

TABLE 2: Hydrolysis with Different Enzymes

Enzyme	ACE-I activity [g/L](IC_{50})	pH	Degree of hydrolysis [%]
Protease P6, neutral protease, *Aspergillus* strains, Amano Enzymes	0.274	7.0	8.9
Protease A, neutral protease, *Aspergillus oryzae*, Amano Enzymes	0.443	7.0	9.2
Protease M, acid protease, *Aspergillus oryzae*, Amano Enzymes	0.450	4.0	7.4
Peptidase, neutral peptidase, *Aspergillus oryzae*, Amano Enzymes	0.540	7.0	6.9
Neutrase, neutral bacterial protease, *Bacillus subtillis*, Novo Nordisk DK	0.510	7.0	4.3
Validase (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.510	4.0	5.6
AFP 2000 (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.550	4.0	3.9

Example 15

Identification of ACEI-Peptides and Measuring their Activities

200 mg of permeate from example 3 was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a Jupiter preparative reverse-phase HPLC column (10 micron, C18, 22 x 250 mm [Phenomenex NZ]) equilibrated with solvent A (0.1%TFA) and connected to an FPLC system (Pharmacia). Peptides were sequentially eluted from the column with a gradient of 0 to 43% solvent B (0.08% TFA in acetonitrile) in 245 min at a flow rate of 10mL/min. Peptides eluting from the column were detected by monitoring the absorbance of the eluate at 214nm. The eluate was collected by an automatic fraction collector set to collect 3 min fractions.

Each fraction was lyophilised and the amount of peptide material present was measured gravimetrically. Fractions were assayed for ACE-I activity using an *in vitro* assay system (reagents from Sigma product 305-10) consisting of rabbit lung ACE and the

colorimetric ACE substrate furylacryloylphenylalanylglycylglycine (FAPGG); ACE hydrolyses FAPGG to give the products FAP and GG which results in a decrease in absorbance at 340mm. If a peptide inhibits ACE, the change in absorbance at 340nm is reduced. Fractions containing the highest ACE inhibitory activity per mg peptide material were re-applied to the preparative reverse-phase HPLC column and eluted using a shallow gradient of solvent B *i.e.* 0.09% increase in solvent B concentration/min. The eluate was collected using the fraction collector set to collect 0.5 min fractions.

Samples from each fraction were analysed using an analytical reverse-phase HPLC column, and those fractions containing a single, identical peptide were pooled. Each pooled fraction was lyophilised and the weight of the peptide present was determined gravimetrically. The purified peptides were assayed for ACE-I activity as before and the IC_{50} was calculated for each individual peptide.

The molecular weight of each peptide was determined by Electrospray Ionisation Mass Spectrometry (Sciex API 300 triple quadrupole mass spectrometer). Tandem mass spectrometry was also done for each peptide to generate CAD spectra using MSMS experiment scans. Each peptide was also analysed by an automated N-terminal sequencer (ABI model 476A protein sequencer). Data collected from all three techniques was used to deduce the sequence of all of the peptides possessing ACE-I activity. The origin of each of the active peptides was determined by searching a database containing the known sequences of all bovine milk proteins.

The peptides, their origins, activities and known similarities are set out in table 3. Although the last three peptides are of a casein origin they were from a whey protein hydrolysate. The rennet used to precipitate casein did not precipitate these casein fractions and they remained with the whey proteins.

TABLE 3: ACE-I Peptides and their Activities

Peptide Sequence[a]	Origin	Activity[b] (IC_{50} in mg L^{-1})	Similarity to known ACE-I Peptides
AFE (Ala-Phe-Glu)	PP[d]3(129-131)	20	
LFSH (Leu-Phe-Ser-His)	PP3(125-128)	30	
ILKEKH (Ile-Leu-Lys-Glu-Lys-His)	PP3(71-76)	20	
LIVTQ (Leu-Ile-Val-Thr-Gln)	β-LG[e](1-5)	17	
MKG (Met-Lys-Gly)	β-LG(7-9)	24	
LDIQK[c] (Leu-Asp-Ile-Gln-Lys)	β-LG(10-14)	17	β-LG(9-14)
VF (Val-Phe)	β-LG(81-82)	19	
ALPMH (Ala-Leu-Pro-Met-His)	β-LG(142-146)	12	β-LG(142-148)
VTSTAV (Val-Thr-Ser-Thr-Ala-Val)	GMP[f](59-64)	30	
LHLPLP (Leu-His-Leu-Pro-Leu-Pro)	β-CN[g](133-138)	7	
LVYPFPGPIPNSLPQNIPP (Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro)	β-CN(58-76)	19	β-CN(74-76)
LFRQ (Leu-Phe-Arg-Glu)	α_{s1}-CN(136-139)	17[h]	

[a] sequence given using the single-letter amino acid code with the corresponding three-letter code in brackets

[b] using the colorimetric substrate FAPGG

[c] most abundant ACE-I in hydrolysate

[d] protease peptone

[e] β-lactoglobulin

[f] glycomacropeptide

[g] β-casein

[h] activity measured with that of another peptide of unknown origin

Example 16

The effect of the hydrolysate powder prepared in example 3 (without ultrafiltration) on *in vivo* blood pressure was tested using spontaneously hypertensive rats (SHR/N). The rat strain has been specifically selected for their development of high blood pressure on maturing, and is used extensively to monitor the effect of blood pressure lowering agents. They were purchased from Animal Resources Centre, P O Box 1180 Canning Vale, Western Australia 6155.

Eight week old rats were individually housed in plastic rat cages and kept in temperature controlled facilities throughout the trial. They had unlimited access to water and were fed commercial rat chow *ad libitum*. The test products were given orally as a single daily dose for 8 weeks during which time changes in blood pressure were monitored. Their blood pressure was measured using a specially designed tail cuff and blood pressure monitoring apparatus (IITC Inc., Life Science Instruments, 23924 Victory Blvd, Woodland Hilld, CA 91367). The experimental design was approved by the Massey University Animal Ethics Committee, protocol number 98/141.

The changes in the systolic blood pressures of each group of animals over the eight weeks are plotted in Figure 2 (as least squares means). The hydrolysate at both 2g/Kg bodyweight/day and 4g/Kg bodyweight/day significantly lowered the systolic blood pressure of SHRs compared to animals fed commercial rat chow only ($p<0.004$ by least-squares means analysis, see Figure 3). The effect of the hydrolysate was not as great as that of captopril, a known ACE-I inhibitory drug administered at 30mg/Kg bodyweight/day, but was a significant improvement for animals fed commercial rat chow only.

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CLAIMS

1. A process for preparing a whey protein hydrolysate containing bioactive peptides which comprises the following steps:

   (i) treating a whey protein containing substrate with one or more enzymes capable of hydrolysing whey proteins, to produce a whey protein hydrolysate containing bioactive peptides; and

   (ii) terminating the hydrolysis before substantial production of unacceptable bitter flavours.

2. A process as claimed in claim 1, wherein the hydrolysis is terminated under conditions which cause no more than partial denaturation of the whey proteins and which maintain or improve their organoleptic properties.

3. A process as claimed in claim 1 or 2, wherein the hydrolysis is terminated under conditions which partially denature the whey proteins and thereby improve their organoleptic properties.

4. A process as claimed in any one of claims 1 to 3 wherein the substrate is sweet whey or sweet whey protein concentrate.

5. A process as claimed in any one of claims 1 to 4 wherein the enzyme capable of hydrolysing the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined).

6. A process as claimed in claim 5 wherein the enzyme is neutrase, and the hydrolysis is carried out at a temperature of between about 40°C and 65°C and at a pH of between about 6 and 7.5.

7. A process as claimed in claim 5 or 6 wherein the hydrolysis is terminated by high temperature short duration heat treatment.

8. A process as claimed in claim 7 wherein said heat treatment is for a period of 1 to 10 seconds at a temperature of about 85°C to about 100°C.

9. A process as claimed in claim 7 wherein the heat treatment is for a period of about 3 seconds at a temperature of about 86°C to 88°C.

10. A process as claimed in any one of claims 7 to 9 wherein the heat treatment is such that the mean particle size of the whey proteins in the hydrolysate following the heat treatment is less than about 30 microns.

11. A process as claimed in claim 10 wherein said resulting mean particle size is less than about 3 microns.

12. A process as claimed in any one of the preceding claims wherein the degree of hydrolysis of the substrate before termination of hydrolysis is below about 10%.

13. A process as claimed in claim 12 wherein the degree of hydrolysis is about 3-5%.

14. A process as claimed in any one of the preceding claims wherein the substrate also contains lactose, in an amount of about 5% by weight or higher.

15. A process as claimed in any one of the preceding claims wherein said lactose content is about 10% by weight or higher.

16. A process as claimed in claim 14 or 15 wherein the amount of lactose present in the substrate is up to about 30% by weight.

17. A process as claimed in any one of claims 14 to 16 wherein the amount of lactose present in the substrate is up to about 50% by weight.

18. A process as claimed in any one of claims 14 to 17, wherein the substrate is also treated with lactase and/or β-galactosidase, either before, during or after the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

19. A process as claimed in claim 6 or in any claim dependent from claim 6 wherein one or more of the bioactive peptides in the resulting hydrolysate is selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

20. A non-bitter product produced by partial hydrolysis of a substrate containing whey proteins, wherein the product contains bioactive peptides and has a degree of hydrolysis of the whey proteins of below about 10%.

21. A product as claimed in claim 20 wherein the degree of hydrolysis of the whey proteins is about 3 to 5%.

22. A product as claimed in either of claims 20 or 21 wherein the mean particle size of the whey proteins in the product is less than about 30 microns.

23. A product as claimed in claim 22 wherein said mean particle size is less than about 3 microns.

24. A product as claimed in any one of claims 20 to 23 which is substantially white in appearance.

25. A product as claimed in any one of claims 20 to 24 which also contains galacto-oligosaccharides.

26. A product as claimed in any one of claims 20 to 25 wherein one or more of the bioactive peptides is selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

27. A food product comprising a product as claimed in any one of claims 20 to 26.

28. A food product containing a whey protein hydrolysate produced by a process as claimed in any one of claims 1 to 19.

29. Any one or any combination of two or more of the bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

30. A method of reducing systolic blood pressure in a subject which comprises administering to that subject an effective amount of a product as claimed in any one of claims 20 to 29.

31. A method as claimed in claim 30 which comprises administering an effective amount of a product according to claim 26.

32. A method as claimed in claim 30 which comprises administering an effective amount of a peptide or a combination of peptides as claimed in claim 29.

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(57) Abstract

The invention relates to a partial hydrolysate of whey protein which contains bioactive peptides but does not have a bitter flavour. The hydrolysate is carried out using selective enzymes which produce the active peptides and is terminated at a degree of hydrolysis before substantial bitter flavours are created. There are also described novel peptides and a method of reducing systolic blood pressure through the administration of the peptides.

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BIOACTIVE WHEY PROTEIN HYDROLYSATE

TECHNICAL FIELD

This invention relates to a process for producing hydrolysed whey protein products which are free of bitter flavours and which contain bioactive peptides. The products of the process have high digestibility and good organoleptic properties. The products may have either a bland or slightly sweet taste and are free of soapy or brothy flavours. The hydrolysed whey protein products may optionally contain oligosaccharides and are useful sources of bioactive peptides for incorporation into functional foods.

BACKGROUND ART

A number of food ingredients and foodstuffs have been produced from the hydrolysis of a protein source such as the milk proteins, casein and whey proteins.

Hydrolysed protein foodstuffs may have advantages over non-hydrolysed protein foodstuffs in a number of areas of health care. For example, it is known that enzymatically hydrolysed proteins are less allergenic. They are also more rapidly digested and absorbed than whole proteins. Foodstuffs containing hydrolysed proteins are also useful in the alimentation of hospital patients with digestive diseases for example.

Hydrolysis of whey proteins and caseins is known to release bioactive peptides that can exhibit a number of physiological effects (Maubois et al, 1991; EP 475506). A number of publications describe such bioactive peptides, for example, ACE inhibiting peptides which have antihypertensive properties have been released through an enzymatic treatment of bovine β-lactoglobulin and whey protein concentrates (Mullally et al, 1997). ACE inhibitory peptides are also found in sour milk and in hydrolysates of α_s and β casein (JP 4282400; Nakamura et al 1994, Yamamoto et al 1994).

EP 4745506 discloses the hydrolysis of the milk protein lactoferrin in whey to release lactoferricin which acts as an antimicrobial agent useful for treating diarrhoea, athlete's foot, eye infections, mastitis etc in humans and animals.

However, the hydrolysis of most food proteins, especially the hydrolysis of whey and casein containing products, is known to generate bitterness. This causes palatability

problems particularly when attempting to formulate orally ingestible products incorporating milk protein hydrolysates as a source of bioactive peptides.

In the field of protein hydrolysis one or both of two approaches are commonly used for controlling or removing bitterness in protein hydrolysates to increase palatability of the products.

The extensive hydrolysis of the protein substrate is known to reduce bitterness in milk protein hydrolysates (EP 065663; EP 117047; US 3970520). Less bitter products are produced relatively easily and cheaply in this way. However, extensive hydrolysis reduces the chain lengths of all peptides, including the bioactive peptides of interest . Extensive hydrolysis of the protein substrate destroys the functional and biological activity of the peptide of interest. In addition soapy and brothy off-flavours often develop, with the consequence that the palatability of the final product remains poor compared to the original bland tasting protein substrate. A final disadvantage is that for some hydrolysates the bitterness is only partially removed (Roy 1992 and 1997).

A second common method for the control of bitterness in protein hydrolysates is to use debittering enzymes, in particular those sourced from *Aspergillus oryzae*.

"Bitterness" generation in protein hydrolysis is thought to be due to the presence of large hydrophobic 'bitter' peptides. Debittering enzymes selectively hydrolyse bitter peptides present in the protein hydrolysates. A worker skilled in the art can - by the judicious selection of debittering enzymes and the conditions of treatment - effectively debitter milk protein hydrolysates leaving intact the particular bioactive peptides of interest. However, use of debittering enzymes makes the process more expensive, and preservation of some of the bioactive peptide is not easily or sucessfully achieved. A further disadvantage is that debittering enzymes treatments have a tendency to release free amino acids into the final product and, as a consequence, the hydrolysates develop unpleasant brothy or soapy flavours (Roy 1992 and 1997).

The various methods of debittering the protein hydrolysates result in additional process steps and add costs to the manufacture of the final product. In addition the final product also becomes overbalanced in its supply of free amino acids.

It would be most advantageous if a process for hydrolysing protein could be developed which releases bioactive peptides of interest and which limits the formation of bitter

peptides and free amino acids, thereby allowing the original bland taste of the milk proteins substrates to be retained.

Some bioactive peptides - in particular the antihypertensive peptides - are relatively stable during protein hydrolysis and are released very early during the hydrolysis of the milk protein substrate as shown in Figure 1.

The bitter flavours of milk protein hydrolysates can be improved by adding sugars or by hydrolysing natural sugars, such as lactose, already present in the milk protein substrate (Bernal and Jelen, 1989). For example sour wheys and cheese wheys are made more palatable when they have been sweetened by β-galactosidase and lactase hydrolysis of lactose (FR 2309154; US 4358464; JP 8056568).

In order to achieve a high flavour acceptability for a hydrolysed protein product which contains bioactive peptides, precise control of hydrolysis is required to prevent bitterness occurring.

A common method of termination of hydrolysis is by deactivation of the enzymes, usually by thermal deactivation at high temperatures, typically > 90-100°C for an extended period of time. However, this method cannot be used to stop the hydrolysis of whey proteins as any intact unhydrolysed whey proteins remaining in the mixture would denature and precipitate making the final product less soluble and less acceptable for the use as a food ingredient.

It would be advantageous if a process of hydrolysing whey protein could be controlled so that it directly produced a hydrolysate comprising bioactive peptides for incorporation into functional foods which did not taste bitter and where the enzyme inactivation steps did not compromise the integrity of the intact proteins in the final product.

It is an object of the invention to go some way towards achieving these desiderata or at least to offer the public a useful choice.

SUMMARY OF THE INVENTION

Accordingly the invention may be said broadly to consist in a process for preparing a whey protein hydrolysate containing bioactive peptides which comprises the following steps:

(i) treating a whey protein containing substrate with one or more enzymes capable of hydrolysing whey proteins, to produce a whey protein hydrolysate containing bioactive peptides; and

(ii) terminating the hydrolysis before substantial production of bitter flavours.

Preferably the hydrolysis is terminated under conditions which cause no more than partial denaturation of the whey proteins and which maintain or improve their organoleptic properties.

More preferably the hydrolysis is terminated under conditions which partially denature the whey proteins and thereby improve their organoleptic properties.

Preferably the enzyme capable of hydrolysing the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined).

Preferably the hydrolysis is terminated by heat treatment, preferably for a period of about 1 to 10 seconds at a temperature of about 85°C to 100°C.

Preferably the degree of hydrolysis of the substrate before termination of hydrolysis is up to 10%.

More preferably the degree of hydrolysis is from about 3% to about 5%.

Preferably the substrate also contains lactose, in an amount of up to 50% by weight.

Alternatively, the substrate also contains lactose in an amount of up to 30% by weight.

Preferably the substrate is also treated with lactase and/or β-galactosidase, either before or during the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

In another embodiment the invention consists in a whey protein hydrolysate containing one or more bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

The enzyme hydrolysis step may be carried out under conditions which are suitable for the particular enzyme used as would be understood by a person skilled in the art.

The whey protein substrates are hydrolysed at a concentration in the range from 5-50% solids and the enzyme or enzyme mixtures are added to give an enzyme to substrate ratio between 0.01% and 3% w/w total solids, preferably between 0.01% and 1.0% w/w total solids.

Protein substrates treated with acid proteases may be hydrolysed at pH between 2.5 and 6.0, preferably between pH 3.0 and 5.0.

Protein substrates treated with neutral proteases may be hydrolysed at pH between 3.5 and 9.0, preferably between pH 6.0 and 8.0.

Protein substrates treated with alkaline proteases may be hydrolysed at pH range between 5 and 10.0, preferably between pH 6.0 and 8.0.

The protein hydrolysis may be carried out at a temperature range of from 20-65°C, preferably from 50-60°C.

The hydrolysis of lactose may be carried out at a prior stage to the whey protein hydrolysis, concurrently therewith or subsequently. The enzymes used for lactose hydrolysis may comprise lactase and/or β-galactosidase and may be selected from yeast or fungal sources eg *Klyvermyces lactis*, *Saccharomyces lactis*, *Saccharomyces fragillis*, eg *Aspergillus niger*, *Aspergillus oryzae* such as Maxilact (Gist Brocades) and Novolact (Novo Nordisk). The lactose hydrolysis is carried out under conditions which would be known to persons skilled in the art.

In one embodiment termination of the hydrolysis is achieved by deactivating the one or more whey protein hydrolysis enzymes (and/or the lactose hydrolysing enzymes added previously) by firstly changing the pH of the reaction mixture to a pH in which the enzyme(s) is either inactive or less active, and/or heating the reaction mixture to a comparatively mild temperature using a heat exchanger to denature the enzyme but not the intact whey proteins in the substrate. A suitable temperature range which would denature the enzymes is in the order of 55-70°C, preferable 65°C.

According to one option, depending on the enzyme(s) used, the enzyme or enzyme mixture may also be deactivated by the evaporation and drying procedures.

According to another option the enzyme or enzyme mixture may also be deactivated with or without a prior pH change.

Alternatively, the one or more enzymes used to selectively hydrolyse the whey protein may be immobilised on an inert support such as Roehm Eupergit, Carrageenan particles, chitosan particles or any other suitable material and then used in a stirred tank or fixed bed reactor or on a membrane or on a hollow fiber reactor.

Alternatively, the enzyme(s) to be used for the hydrolysis could be cross linked to suitable inert support prior to the hydrolysis reaction and subsequently separated out of the hydrolysis reaction with the use of a microfiltration membrane.

Alternatively, the enzyme can be separated away from the hydrolysis mixture with the use of an ultrafiltration membrane with a nominal molecular weight cutoff in the range 10 - 500 kDa once hydrolysis is complete.

After hydrolysis and optional deactivation or removal of enzymes, the hydrolysate may optionally be subjected to reverse osmosis under conditions whereby salt and water are removed from the hydrolysate. The purified desalted hydrolysate comprising whey proteins and bioactive peptides is then recovered. If lactose hydrolysis is also chosen then the hydrolysate will also contain glucose, galactose and/or galacto-oligosaccharides.

Optionally the hydrolysed whey proteins containing the bioactive peptide fraction can be separated with a UF membrane of 5-200 kDa cut off, preferably 10-50 kDa cut off. The bioactive peptides, other peptides and, optionally, hydrolysed lactose is recovered in the permeate.

According to another option ion exchange or hydrophobic adsorption or hydrophobic interaction chromatography or combinations of these processes may be used to recover the hydrolysed bioactive fraction from the hydrolysates in an enriched form.

In addition, lactase and β-galactosidase hydrolysis of lactose produces galacto-oligosaccharides which are known to stimulate the growth of beneficial gut flora thereby adding to the bioactive properties of the hydrolysates.

Hydrolysates which have been treated to further hydrolyse lactose are useful as food additives for consumers who are lactose intolerant.

The hydrolysed whey protein product of the invention has one or more of the following features:

- antihypertensive ACE-I activity
- bifidus growth promoting activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste
- good organoleptic properties.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described with reference to the accompanying drawings in which:

Figure 1 is a plot of bitterness and bioactivity on the ordinant against the degree of hydrolysis on the abscissa. The 'opportunity window' of obtaining a product according to the present invention containing bioactive peptides and having acceptable flavours before the hydrolysis reaction produces bitter peptides is between the lines x_1 and x_2.

Figure 2 is a plot of systolic blood pressure of four groups of hypertensive rats fed different diets over a period of eight weeks.

Figure 3 is a plot of a least squares means analysis of rats fed with a control of commercial rat chow against groups of rats fed with hydrolysate at two different concentrations per day.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention provides a process for producing a hydrolysed whey protein product containing bioactive peptides, whereby the hydrolysis is carried out under a high degree of control to prevent undesirable flavours developing during hydrolysis (eg bitter, soapy and brothy). The hydrolysis is terminated within the "opportunity window", ie before the emergence of substantial bitterness - as shown in Figure 1 - to provide hydrolysates having good organoleptic properties and maximum bioactive peptides. In Figure 1 the degree of hydrolysis is represented qualitatively on

the x axis. The window of opportunity is between the points x_1 and x_2 which will vary depending on the enzyme which is used. The optimum conditions sought are a maximum bioactivity with an acceptable level of bitterness.

In particularly preferred embodiments of the process of the invention, the enzyme which hydrolyses the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined) and the hydrolysis of the whey proteins is terminated by heat treatment for a short time at a high temperature (about 85-100°C for 1-10 seconds). The applicants have surprisingly found that the above enzymes (1) are able to produce a whey protein hydrolysate containing a good level of bioactive peptides, and (2) can be inactivated by a short time, high temperature treatment which causes only partial denaturation of the whey proteins in the hydrolysate, and surprisingly improves the organoleptic properties of the whey proteins, in terms of providing a product which is creamy in texture (has a relatively small particle size) and substantially white in appearance.

The present invention is now exemplified by the following examples:

Example 1

A 10% solution of a sweet whey protein concentrate with 80% protein content (ALACEN™ 392, 2L) was hydrolysed at 50°C with the commercially available enzyme Neutrase sourced from *Bacillus subtilis* (Novo Nordisk, Denmark). A pH of 7.0 and an enzyme substrate ratio of 0.3% w/w was used for the hydrolysis. The hydrolysate was adjusted to pH 5.0 and heated at 65°C for 30min to inactivate the enzyme. The hydrolysate (DH of 4.5%) was spray dried and tested for angiotensin-converting enzyme inhibitor (ACE-I) activity and flavour. ACE-I activity in the dried product was determined using FAPGG as a substrate (Product 305-10 ex Sigma Chemical Corporation, St Louis, MO, USA) according of the method of D W Cushman & H S Cheung (1971). ACE-I activities are expressed as the amount of material (g/L) needed to reduce the activity of the ACE-I enzyme by 50%. IC_{50} ACE-I activity in the hydrolysate was 0.44g/L and flavour acceptability score, as determined by a taste panel, was very high.

Example 2

A 50% solution of ALACEN™ 421 whey protein concentrate (56% protein content, 10L) was treated with commercial lactase sourced from *Kluveromyces lactis* (Lactozyme

3000L ex Novo Nordisk) at an enzyme to substrate ratio of 0.3% at 50°C for 2 hours. The lactase treated solution was hydrolysed with Neutrase (Novo Nordisk, Denmark) for 1 hour at 50°C at an enzyme substrate ratio of 0.3%. Active enzymes were inactivated by UHT treatment (5sec at 95°C) after a five fold dilution of the mixture. The hydrolysate was spray dried. The dry powder (DH 2.8%) contained no traces of active enzyme and had an ACE-I activity of 2.18g/L. The flavour score was exceptionally high due to the introduction of a low level of sweetening into the product. ACE-I measurements and flavour acceptability scoring were determined as for Example 1.

Example 3

A 500L hydrolysate, made from ALACEN™ 392 in a similar way to that in example 1, was cooled to 10°C after enzyme inactivation. A sub-sample of the original hydrolysate was dried. The remaining hydrolysate was subjected to ultrafiltration at 10°C with a 10,000 dalton nominal molecular weight cutoff membrane (HFK 131, Koch Membrane Systems, USA). The hydrolysate (at a DH between 3.8% and 4.2%) was concentrated to a VCF 10 and the retentate was dried directly. The permeate was concentrated by evaporation to approx 25% solids and dried. ACE-I measurement and flavour acceptability scoring were determined as for Example 1. The ACE-I activity was enriched in the permeate powder (IC_{50} of the permeate powder was 0.15g/L). ACE-I activity in the sub-sample of the dried hydrolysate before ultrafiltration was 0.43g/L. The flavour acceptability scores on the retentate powder and the spray dried powder of the feed were both high.

Example 4

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up at 15% solids to yield 150 L. The solution was treated with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) and a commercial lactase from *Klyvermyces lactis* (Lactozyme 3000L ex Novo Nordisk). The addition rate of enzyme was 0.3% w/w (on protein basis) for Neutrase and 1.2% w/w (on lactase basis) for Lactozyme. The reaction continued for 2h at 50°C at a pH of 7.0. Samples of 35L were taken every 0.5h inactivated at 88°C for 3 seconds and subsequently spray dried. The ACE-I activity as specified in example 1 yielded 0.424g/L, 0.336g/L and 0.432g/L for the three mixtures on a protein basis. The bitterness of the samples from ALACEN™ 392 was formally evaluated against two standard hydrolysates. The scores for bitterness on a scale of 1 to 10, 10 being most

bitter were 1.9 for a sample after 0.5h hydrolysis, 2.3 for the 2h hydrolysis compared to 5 and 7 for the standard hydrolysis samples of greater degrees of hydrolysis.

The samples of ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose taken after 2h had a mean particle size of 3μm or 2μm respectively. The sample of ALACEN™ 392 had a mean particle size of 6μm after 2h hydrolysis and inactivation as specified. Less grittiness and chalkiness was attributed to the smaller particle size samples.

The solubility of the hydrolysed ALACEN™ 392/lactose mixture was the highest with approximately 85% across the pH range. The ALACEN™ 392, ALACEN™ 421 samples are soluble to about 70% with a slight drop in solubility to 65% at pH 3.5.

Example 5

Three different solutions from ALACEN™ 392, ALACEN™ 421 and a mixture of ALACEN™ 392 and lactose were made up of 30% solids to yield 75L. The enzyme treatment was done using the same conditions as example 4. The samples taken at half hourly intervals were diluted to 15% solids. Otherwise the heat treatment was done as in example 4. The ACE-I activity measured as specified in example 1 was 0.560g/L, 0.440g/L and 0.728g/L.

Samples from example 4 and 5 were added in a concentration of 0.1% to the standard growth media of *Bifidobacterium lactis* and resulted in a faster cell growth and higher final cell density of the strain than the control without any supplement.

The oligosaccharide level (trisaccharides and higher) of those three hydrolysed samples was 0.2%, 2.1% and 7.0% in ALACEN™ 392, ALACEN™ 421 and the mixture of ALACEN™ 392 and lactose, respectively.

Example 6

Hydrolysate powders prepared in example 5 were used as a supplement for yoghurts in addition rates from 2.5% and 5% of the final yoghurt and resulted in an increased creaminess and improved the texture compared to the control.

Example 7

The hydrolysate powders prepared in example 5 were used as the protein source in a muesli bar recipe on a 6% and 12% w/w addition rate. All tasters preferred the hydrolysate bars over the unhydrolysed WPC control. The best results were achieved with hydrolysed ALACEN™ 421 and a mixture of ALACEN ™392 and lactose prepared in example 5.

Example 8

The hydrolysate powder prepared in example 5 was used as an ingredient in a meal replacer concept sample. ALACEN™ 421 hydrolysed in lactose and protein was added at a rate of 45% w/w to whole milk powder, malto dextrin, sucrose and milk calcium (ALAMIN™) to result in a powder meal replacer prototype. In comparison with a control sample without hydrolysed whey protein, hydrolysed whey protein prepared in example 5 was found to be more acceptable.

Example 9

A nutritional whey protein drink was formulated containing 8% w/w of ALACEN™ 392 or ALACEN™ 421 or a mixture of ALACEN™ 392 and lactose hydrolysed as specified in example 5. The drink also contained sucrose, citric acid, flavouring and colouring agents. The pH of the drink was adjusted to 4.3. The drink combined the nutritional and health advantages of whey protein with the refreshing taste of a soft drink. Compared to a drink containing untreated whey protein control the pH stability was improved and the drink had a more milk like appearance than the control.

Example 10

A further nutritional protein drink was formulated containing 12.5% w/w of ALACEN™ 421 hydrolysed as in example 5 in water mixed with pasteurised whole milk. Sucrose was added to yield 6% of the final formulation as well as stabiliser. The drink was flavoured when desired with banana, vanilla or similar flavours. To achieve an extended shelf life the drink was ultra high heated to 140°C for 3 seconds. The mean particle size remains at 3 microns after the additional UHT heat treatment.

Example 11

The hydrolysis was carried out as specified in example 5 but instead of reconstituting ALACEN™ 421 powder a fresh retentate of ALACEN™ was concentrated to 30% solids in the solution. The neutrase addition rate was varied to 0.9% w/w (on a protein base), the lactase level as specified. The reaction mixture was inactivated at 15% solids after 2h. The ACE-I activity yielded 0.480g/L. The organoleptic properties, particle size and food application were very similar to example 4 and 5.

Example 12

The hydrolysis was carried out as specified in example 4 with ALACEN™ 421 powder. The Neutrase addition rate was varied to 0.9% w/w (on a protein basis). The lactose was converted with a lactase from *Aspergillus oryzae* (Fungal lactase 30,000, Kyowa Enzymes Co. Ltd. Japan) on an addition rate of 0.4% w/w (on lactose base). The reaction mixture was inactivated after 1.5h with direct steam injection to achieve a temperature of 88°C for either 1.5 seconds or 3 seconds.

The particle size was 2.3 microns. Organoleptic properties and food application were very similar to the product of example 4.

Example 13

A 10% w/w solution of ALACEN™ 392 was hydrolysed with a commercial protease from *Bacillus subtilis* Neutrase (Novo, Nordisk Denmark) at an enzyme concentration of 0.9% w/w. The reaction continued for 6h at 50°C. Samples of 200ml were taken every 1h, inactivated at 88°C for 8 seconds and subsequently freeze dried.

ACE-I activity, degree of hydrolysis, pH of solution and bitterness developed over time as follows. The higher the bitterness score the more bitter is the taste. The smaller the level measured, the higher is the ACE-I activity.

TABLE 1: Hydrolysis of ALACEN™ 392 WPC

Hydrolysis time [h]	ACE-I activity [g/L] (IC_{50})	Degree of hydrolysis [%]	pH of solution	Bitterness score [informal, 0-10]
1	0.420	3.86	7.01	0
2	0.280	3.78	6.96	1
3	0.230	4.53	6.92	1
4	0.220	4.89	6.89	3.5
5	0.210	5.20	6.87	2
6	0.190	5.37	6.87	4.5

Example 14

A 10% w/w solution of ALACEN™ 392 was hydrolysed with the following commercial proteases at 1% w/w, 50°C for 1h. The reaction was inactivated at 88°C for 8 seconds and subsequently the hydrolysate was freeze dried.

TABLE 2: Hydrolysis with Different Enzymes

Enzyme	ACE-I activity [g/L](IC_{50})	pH	Degree of hydrolysis [%]
Protease P6, neutral protease, *Aspergillus* strains, Amano Enzymes	0.274	7.0	8.9
Protease A, neutral protease, *Aspergillus oryzae*, Amano Enzymes	0.443	7.0	9.2
Protease M, acid protease, *Aspergillus oryzae*, Amano Enzymes	0.450	4.0	7.4
Peptidase, neutral peptidase, *Aspergillus oryzae*, Amano Enzymes	0.540	7.0	6.9
Neutrase, neutral bacterial protease, *Bacillus subtillis*, Novo Nordisk DK	0.510	7.0	4.3
Validase (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.510	4.0	5.6
AFP 2000 (Genancor), acid fungal protease, *Aspergillus niger*, Enzyme Services Ltd. NZ	0.550	4.0	3.9

Example 15

Identification of ACEI-Peptides and Measuring their Activities

200 mg of permeate from example 3 was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a Jupiter preparative reverse-phase HPLC column (10 micron, C18, 22 x 250 mm [Phenomenex NZ]) equilibrated with solvent A (0.1%TFA) and connected to an FPLC system (Pharmacia). Peptides were sequentially eluted from the column with a gradient of 0 to 43% solvent B (0.08% TFA in acetonitrile) in 245 min at a flow rate of 10mL/min. Peptides eluting from the column were detected by monitoring the absorbance of the eluate at 214nm. The eluate was collected by an automatic fraction collector set to collect 3 min fractions.

Each fraction was lyophilised and the amount of peptide material present was measured gravimetrically. Fractions were assayed for ACE-I activity using an *in vitro* assay system (reagents from Sigma product 305-10) consisting of rabbit lung ACE and the

colorimetric ACE substrate furylacryloylphenylalanylglycylglycine (FAPGG); ACE hydrolyses FAPGG to give the products FAP and GG which results in a decrease in absorbance at 340mm. If a peptide inhibits ACE, the change in absorbance at 340nm is reduced. Fractions containing the highest ACE inhibitory activity per mg peptide material were re-applied to the preparative reverse-phase HPLC column and eluted using a shallow gradient of solvent B *i.e.* 0.09% increase in solvent B concentration/min. The eluate was collected using the fraction collector set to collect 0.5 min fractions.

Samples from each fraction were analysed using an analytical reverse-phase HPLC column, and those fractions containing a single, identical peptide were pooled. Each pooled fraction was lyophilised and the weight of the peptide present was determined gravimetrically. The purified peptides were assayed for ACE-I activity as before and the IC_{50} was calculated for each individual peptide.

The molecular weight of each peptide was determined by Electrospray Ionisation Mass Spectrometry (Sciex API 300 triple quadrupole mass spectrometer). Tandem mass spectrometry was also done for each peptide to generate CAD spectra using MSMS experiment scans. Each peptide was also analysed by an automated N-terminal sequencer (ABI model 476A protein sequencer). Data collected from all three techniques was used to deduce the sequence of all of the peptides possessing ACE-I activity. The origin of each of the active peptides was determined by searching a database containing the known sequences of all bovine milk proteins.

The peptides, their origins, activities and known similarities are set out in table 3. Although the last three peptides are of a casein origin they were from a whey protein hydrolysate. The rennet used to precipitate casein did not precipitate these casein fractions and they remained with the whey proteins.

TABLE 3: ACE-I Peptides and their Activities

Peptide Sequence[a]	Origin	Activity[b] (IC_{50} in mg L^{-1})	Similarity to known ACE-I Peptides
AFE (Ala-Phe-Glu)	PP[d]3(129-131)	20	
LFSH (Leu-Phe-Ser-His)	PP3(125-128)	30	
ILKEKH (Ile-Leu-Lys-Glu-Lys-His)	PP3(71-76)	20	
LIVTQ (Leu-Ile-Val-Thr-Gln)	β-LG[e](1-5)	17	
MKG (Met-Lys-Gly)	β-LG(7-9)	24	
LDIQK[c] (Leu-Asp-Ile-Gln-Lys)	β-LG(10-14)	17	β-LG(9-14)
VF (Val-Phe)	β-LG(81-82)	19	
ALPMH (Ala-Leu-Pro-Met-His)	β-LG(142-146)	12	β-LG(142-148)
VTSTAV (Val-Thr-Ser-Thr-Ala-Val)	GMP[f](59-64)	30	
LHLPLP (Leu-His-Leu-Pro-Leu-Pro)	β-CN[g](133-138)	7	
LVYPFPGPIPNSLPQNIPP (Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro)	β-CN(58-76)	19	β-CN(74-76)
LFRQ (Leu-Phe-Arg-Glu)	α_{s1}-CN(136-139)	17[h]	

[a] sequence given using the single-letter amino acid code with the corresponding three-letter code in brackets

[b] using the colorimetric substrate FAPGG

[c] most abundant ACE-I in hydrolysate

[d] protease peptone

[e] β-lactoglobulin

[f] glycomacropeptide

[g] β-casein

[h] activity measured with that of another peptide of unknown origin

Example 16

The effect of the hydrolysate powder prepared in example 3 (without ultrafiltration) on *in vivo* blood pressure was tested using spontaneously hypertensive rats (SHR/N). The rat strain has been specifically selected for their development of high blood pressure on maturing, and is used extensively to monitor the effect of blood pressure lowering agents. They were purchased from Animal Resources Centre, P O Box 1180 Canning Vale, Western Australia 6155.

Eight week old rats were individually housed in plastic rat cages and kept in temperature controlled facilities throughout the trial. They had unlimited access to water and were fed commercial rat chow *ad libitum*. The test products were given orally as a single daily dose for 8 weeks during which time changes in blood pressure were monitored. Their blood pressure was measured using a specially designed tail cuff and blood pressure monitoring apparatus (IITC Inc., Life Science Instruments, 23924 Victory Blvd, Woodland Hilld, CA 91367). The experimental design was approved by the Massey University Animal Ethics Committee, protocol number 98/141.

The changes in the systolic blood pressures of each group of animals over the eight weeks are plotted in Figure 2 (as least squares means). The hydrolysate at both 2g/Kg bodyweight/day and 4g/Kg bodyweight/day significantly lowered the systolic blood pressure of SHRs compared to animals fed commercial rat chow only ($p<0.004$ by least-squares means analysis, see Figure 3). The effect of the hydrolysate was not as great as that of captopril, a known ACE-I inhibitory drug administered at 30mg/Kg bodyweight/day, but was a significant improvement for animals fed commercial rat chow only.

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CLAIMS

1. A process for preparing a whey protein hydrolysate containing bioactive peptides which comprises the following steps:

   (i) treating a whey protein containing substrate with one or more enzymes capable of hydrolysing whey proteins, to produce a whey protein hydrolysate containing bioactive peptides; and

   (ii) terminating the hydrolysis before substantial production of unacceptable bitter flavours.

2. A process as claimed in claim 1, wherein the hydrolysis is terminated under conditions which cause no more than partial denaturation of the whey proteins and which maintain or improve their organoleptic properties.

3. A process as claimed in claim 1 or 2, wherein the hydrolysis is terminated under conditions which partially denature the whey proteins and thereby improve their organoleptic properties.

4. A process as claimed in any one of claims 1 to 3 wherein the substrate is sweet whey or sweet whey protein concentrate.

5. A process as claimed in any one of claims 1 to 4 wherein the enzyme capable of hydrolysing the whey proteins is selected from the group consisting of Protease P6, Protease A, Protease M, Peptidase, Neutrase, Validase and AFP 2000 (all as herein defined).

6. A process as claimed in claim 5 wherein the enzyme is neutrase, and the hydrolysis is carried out at a temperature of between about 40°C and 65°C and at a pH of between about 6 and 7.5.

7. A process as claimed in claim 5 or 6 wherein the hydrolysis is terminated by high temperature short duration heat treatment.

8. A process as claimed in claim 7 wherein said heat treatment is for a period of 1 to 10 seconds at a temperature of about 85°C to about 100°C.

9. A process as claimed in claim 7 wherein the heat treatment is for a period of about 3 seconds at a temperature of about 86°C to 88°C.

10. A process as claimed in any one of claims 7 to 9 wherein the heat treatment is such that the mean particle size of the whey proteins in the hydrolysate following the heat treatment is less than about 30 microns.

11. A process as claimed in claim 10 wherein said resulting mean particle size is less than about 3 microns.

12. A process as claimed in any one of the preceding claims wherein the degree of hydrolysis of the substrate before termination of hydrolysis is below about 10%.

13. A process as claimed in claim 12 wherein the degree of hydrolysis is about 3-5%.

14. A process as claimed in any one of the preceding claims wherein the substrate also contains lactose, in an amount of about 5% by weight or higher.

15. A process as claimed in any one of the preceding claims wherein said lactose content is about 10% by weight or higher.

16. A process as claimed in claim 14 or 15 wherein the amount of lactose present in the substrate is up to about 30% by weight.

17. A process as claimed in any one of claims 14 to 16 wherein the amount of lactose present in the substrate is up to about 50% by weight.

18. A process as claimed in any one of claims 14 to 17, wherein the substrate is also treated with lactase and/or β-galactosidase, either before, during or after the whey protein hydrolysis, to hydrolyse the lactose to galactose and glucose and synthesize galacto-oligosaccharides.

19. A process as claimed in claim 6 or in any claim dependent from claim 6 wherein one or more of the bioactive peptides in the resulting hydrolysate is selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

20. A non-bitter product produced by partial hydrolysis of a substrate containing whey proteins, wherein the product contains bioactive peptides and has a degree of hydrolysis of the whey proteins of below about 10%.

21. A product as claimed in claim 20 wherein the degree of hydrolysis of the whey proteins is about 3 to 5%.

22. A product as claimed in either of claims 20 or 21 wherein the mean particle size of the whey proteins in the product is less than about 30 microns.

23. A product as claimed in claim 22 wherein said mean particle size is less than about 3 microns.

24. A product as claimed in any one of claims 20 to 23 which is substantially white in appearance.

25. A product as claimed in any one of claims 20 to 24 which also contains galacto-oligosaccharides.

26. A product as claimed in any one of claims 20 to 25 wherein one or more of the bioactive peptides is selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

27. A food product comprising a product as claimed in any one of claims 20 to 26.

28. A food product containing a whey protein hydrolysate produced by a process as claimed in any one of claims 1 to 19.

29. Any one or any combination of two or more of the bioactive peptides selected from the group consisting of AFE, LFSH, ILKEKH, LIVTQ, MKG, LDIQK, VF, ALPMH, VTSTAV, LHLPLP, LVYPFPGPIPNSLPQNIPP and LFRQ.

30. A method of reducing systolic blood pressure in a subject which comprises administering to that subject an effective amount of a product as claimed in any one of claims 20 to 29.

31. A method as claimed in claim 30 which comprises administering an effective amount of a product according to claim 26.

32. A method as claimed in claim 30 which comprises administering an effective amount of a peptide or a combination of peptides as claimed in claim 29.

ABSTRACT

The invention relates to a partial hydrolysate of whey protein which contains bioactive peptides but does not have a bitter flavour. The hydrolysate is carried out using selective enzymes which produce the active peptides and is terminated at a degree of hydrolysis before substantial bitter flavours are created. There are also described novel peptides and a method of reducing systolic blood pressure through the administration of the peptides.

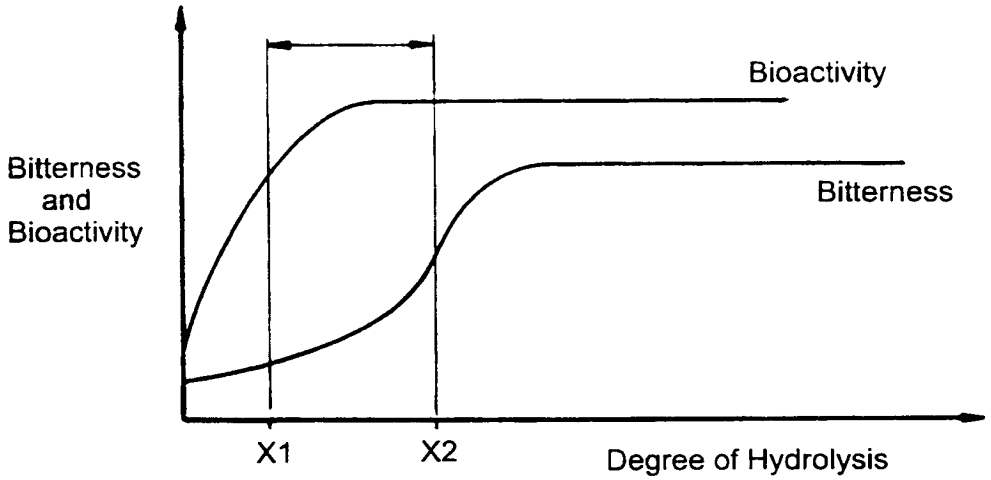
Bioactivity
Bitterness
Bitterness and Bioactivity
X1
X2
Degree of Hydrolysis

FIG. 1

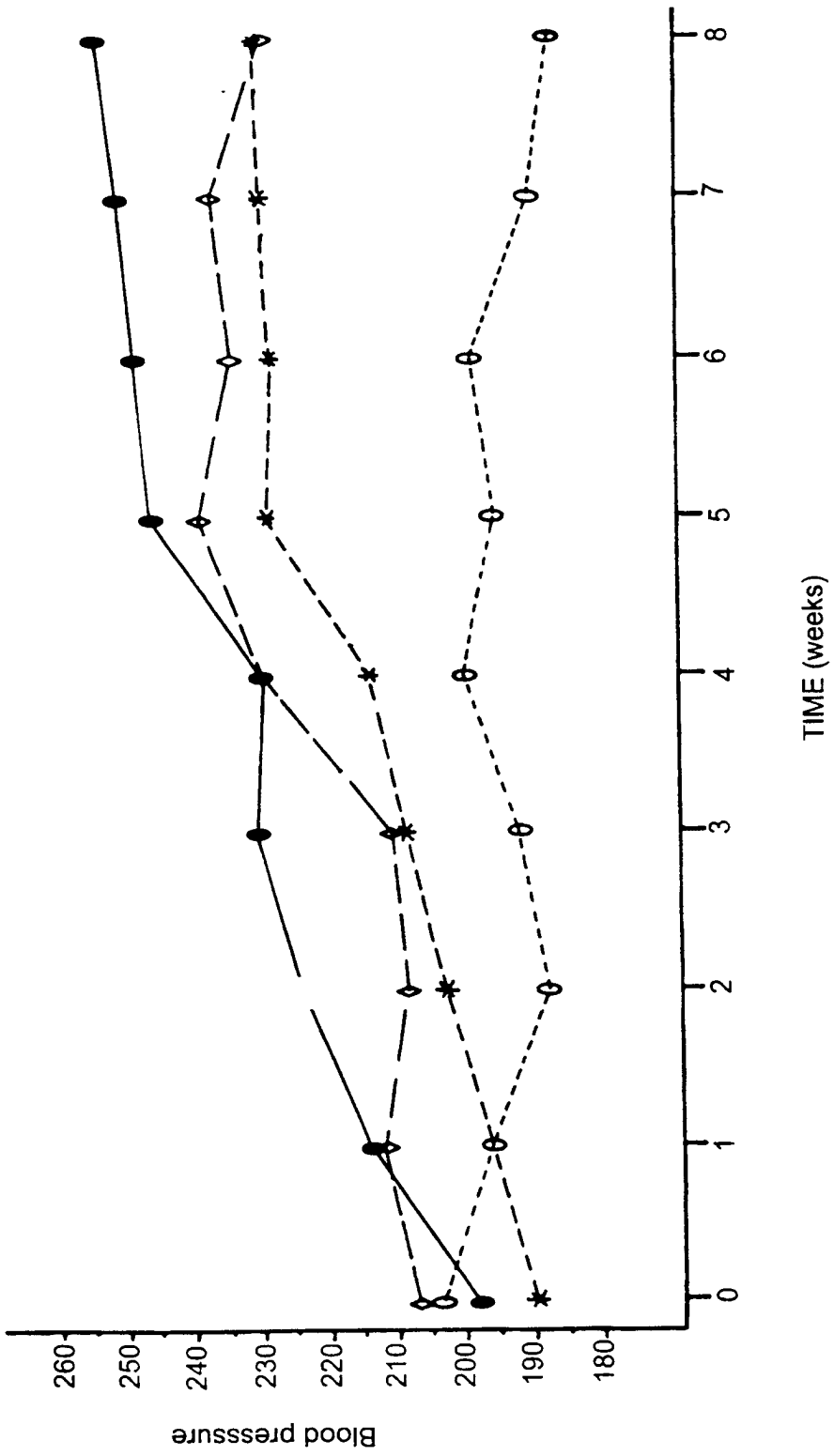
Blood pressure
260
250
240
230
220
210
200
190
180
0
1
2
3
4
5
6
7
8
TIME (weeks)
Control
Hydrolysate(2g/kg bodyweight/day)
Hydrolysate(4g/kg bodyweight/day)
Captopril (30mg/kg bodyweight/day)

FIG. 2

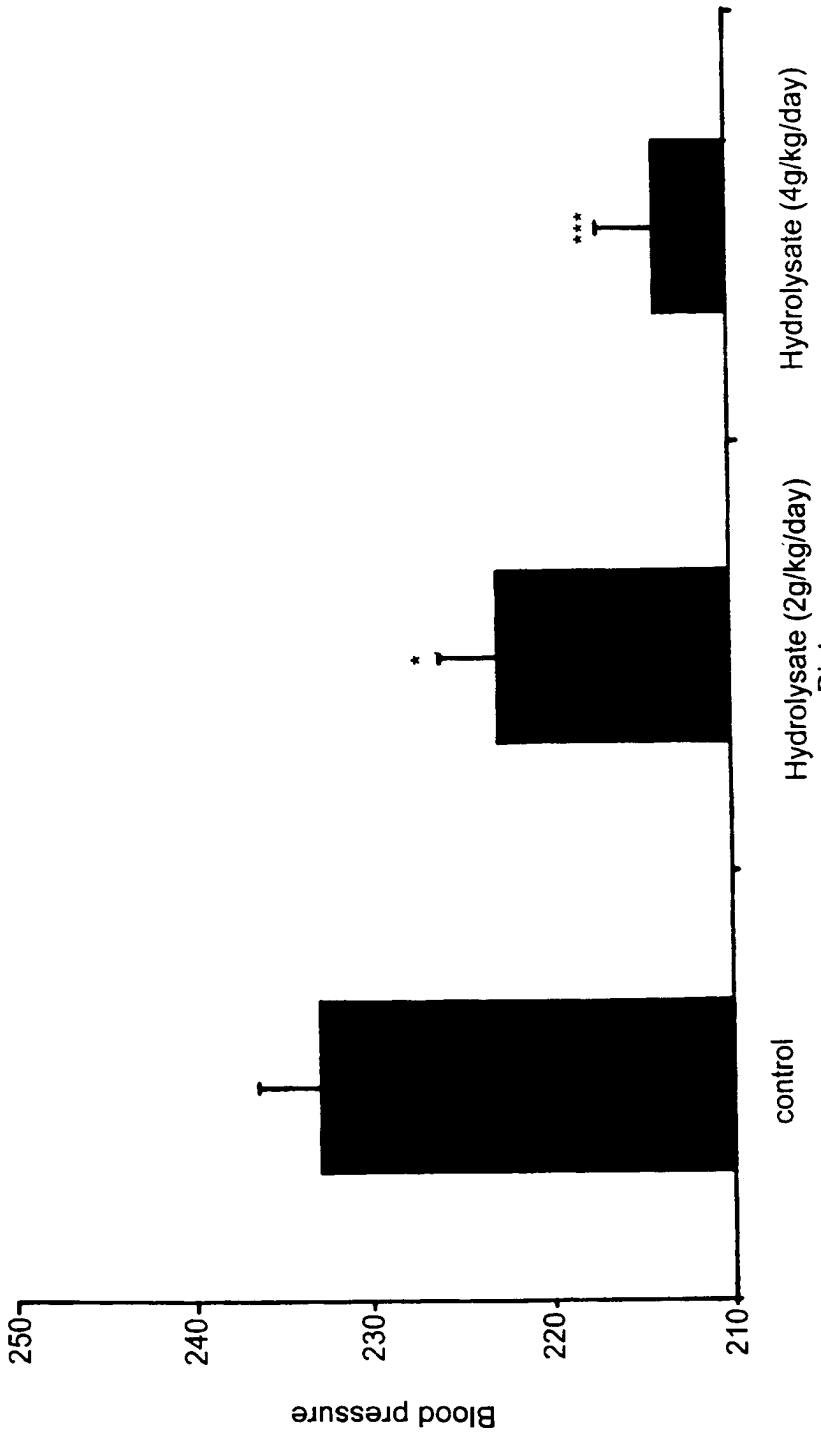
Blood pressure
250
240
230
220
210
control
Hydrolysate (2g/kg/day)
Diet
Hydrolysate (4g/kg/day)
*
***
* p=0.048
*** p=0.0006

FIG. 3